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Investigating the genetics and physiological basis of differences in cadmium and zinc concentrations in tubers of potato (*Solanum tuberosum* L): implications for food safety and biofortification

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The thesis is submitted to University College Cork in fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract

Because Cd is a non-essential element with toxic effects on humans, while the other Group 12 transition element, Zn, is an essential micronutrient for human health, simultaneous selection of reduced Cd and increased Zn, if possible, would be an important objective of potato breeding. To develop cultivars with greater Zn and less Cd concentrations in the tubers, it is important to understand the physiological and genetics mechanisms that gives rise to differences in tuber Cd and Zn concentrations. To achieve these objectives, two potato cultivars, which accumulate larger tuber Cd and Zn concentrations (Lady Rosetta) or smaller tuber Cd and Zn concentrations (Cara), were used in this study.

The patterns of Cd concentration, Cd content and dry weight accumulation of the two cultivars were examined at different stages of plant growth. The data suggest that differences in total Cd uptake and in Cd partitioning among organs are the mechanisms governing differences in tuber -Cd accumulation in the two cultivars. The cultivar exhibiting less concentrations of tuber Cd exhibited less root-to-shoot and shoot-to-tuber translocation, driven by larger root and shoot biomass which retained more Cd in roots and shoots, respectively, reducing its movement to the tubers. Greater remobilization and more efficient tuber loading was observed in the cultivar which accumulated more tuber Cd, indicating that remobilization of Cd from leaves to tubers was a major factor, not only in tuber-Cd loading, but also in the establishment of differences in tuber-Cd. Regardless of cultivar differences, the concentration of Cd in the tuber was very low compared to that in other organs, suggesting that, despite its large phloem mobility, Cd tends to be sequestered in the shoots. Like Cd, the two cultivars differed in Zn concentration, Zn content and dry weight partitioning between organs. The differences in tuber Zn concentration between the two cultivars were associated with differences in internal Zn distribution between stem and tuber. Lady Rosetta translocated more Zn to the tubers while Cara accumulated relatively more Zn in the stems. The greater stem Zn concentration of Cara was associated with greater stem biomass rather than preferential allocation of Zn to the stem. Further examination of reciprocal grafted plants and selected F₁ genotypes derived from a cross between the two cultivars showed that partitioning between tuber and shoot was an important variable for explaining differences in tuber Cd and Zn concentration.

A segregating population, comprising 188 F₁ progeny derived from a cross between the two cultivars, was genotyped using the SolCap 8303 SNP array, and evaluated for Cd, Zn and maturity-related traits. Linkage and QTL mapping were performed using TetraploidSNPMap software, which incorporates all allele dosage information. The final genetic map comprised 3755 SNP markers with an average marker density of 2.94 per cM. Tuber Cd and Zn concentrations were measured in the segregating population over two years. QTL mapping identified four loci for tuber Cd concentration on chromosomes 3, 5, 6 and 7, which explained genetic variance ranging from 5% to 33%, and five loci for tuber Zn concentration on chromosome 1, 3, 5 and, 6, explaining from 5% to 38% of genetic variance. Among the QTLs identified for tuber Cd concentration, three loci coincided with tuber Zn concentrations. The major effect QTL for both tuber Cd and Zn concentration coincided with the maturity locus on chromosome 5, where earliness was associated with increased tuber concentration of both elements. Coincident minor-effect QTLs for Cd and Zn, sharing the same direction of effect, were also found on chromosomes 3 and 6, and these were unrelated to maturity. The results indicate partially overlapping genetic control of tuber Cd and Zn concentration in the cross, involving both maturity-related and non-maturity-related mechanisms.

Declaration

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree of Doctor of Philosophy in UCC, and I have not obtained a degree elsewhere on the basis of the research presented in the submitted work. I declare that this thesis was composed by myself, and the work is my own unless otherwise stated.

Signed.....

Date.....

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Finally, and most importantly, I would like to thank my wife Eleni Kebede for her support, encouragement and unconditional love. I know it has not been easy, but you made possible to take care of our beloved daughter, Yohanna in my absence. My parents, Mulu Tizazu and Fentie Mengist, receive my deepest gratitude and love for your love and encouragement throughout my life. Your prayer for me was what sustained me thus far. To my many family and friends, you should know that your support and encouragement was worth more than I can express on paper. I only say thank you all.

Dedication

This thesis is affectionately dedicated to my wife Eleni Kebede and my
lovely daughter Soliyana Molla

Abbreviation

ANOVA	Analysis of Variance
ATSDR	Agency for Toxic Substances and Disease Registry
BLUE	best linear unbiased estimate
bp	base pairs
Cd	cadmium
cM	centimorgan
CTAB	cetyltrimethylammonium bromide
DAP	days after planting
ddH ₂ O	double-distilled water
DW	dry weight
GS	genomic selection
h	homologous
H ²	broad sense heritability
ICP-MAS	inductively coupled plasma mass spectrometry
LMWOA	low molecular weight organic acid
LOD	logarithms of odds
LR	Lady Rosetta
MAS	marker-assisted selection
Mb	mega base pairs
ML	maximum limit
PC	phytochelatin
PGSC	Potato Genome Sequence Consortium
PQ	partition quotient
QTL	quantitative trait loci
RAPD	random amplified polymorphic DNA
SNP	single nucleotide polymorphism
SolCAP	Solanaceae Agricultural Project
TE	Tris (hydroxymethyl) aminomethane and ethylenediaminetetraacetic acid
Tris	Tris (hydroxymethyl) aminomethane
Zn	zinc
~	approximately

Chapter 1: Background Information

Cadmium (Cd) is a transition metal with an atomic number of 48 and atomic weight of 112. It belongs to group 12, the same group as zinc and mercury. In its pure form, it is characterised as a soft, malleable, tarnishable and silver-white or bluish-white element. Cd can be found in water-insoluble and -soluble forms. The most common water-soluble forms are cadmium chloride (CdCl_2) and cadmium sulphate (CdSO_4) (ATSDR, 2012).

Cd is predominantly found in the earth's crust in association with zinc, lead and copper ores (Clemens, 2006). Natural processes such as weathering and volcanic activities release Cd to the environment. However, these natural processes result in only trace levels of Cd being released to the environment (Clemens, 2006). The main sources of Cd in the environment are smelting, fossil fuel combustion, municipal waste incineration and disposal, and agricultural practices including the application sewage sludge and phosphate fertilisers as Cd occurs naturally in rock phosphate (Clemens, 2006; Kidd *et al.*, 2009; Gallego *et al.*, 2012; Clemens *et al.*, 2013; Roberts, 2014).

An elevated concentration of Cd in the environment can cause serious problems to all organisms including humans. Human exposure to Cd can result in serious health problems including cancer (ATSDR, 2012; Gallego *et al.*, 2012). The long biological half-life of Cd (2-3 decades) and its accumulation in important human organs, such as liver and kidney, can result in chronic health problems following even a minor exposure (Grant *et al.*, 2008; Gallego *et al.*, 2012; Clemens *et al.*, 2013). The main routes of Cd exposures are inhalation, smoking of cigarettes and ingestion of Cd-contaminated food. Cd exposure due to air inhalation is not a major concern except for people living near Cd-emitting industries (ATSDR, 2012). The major source of Cd exposure for non-smokers is from the food supply. The main source of Cd in the food chain is soil Cd that is taken up by the plant roots and accumulated in edible portions (Clemens, 2006; ATSDR, 2012).

With increasing evidence of Cd's toxicity, national and global legislative bodies have set maximum limits of Cd concentration in food chains especially for staple crops. For potato, the maximum Cd concentration limit is 0.1 mg kg^{-1} fresh weight basis as set by several jurisdictions, including Europe (European Commission, 2006), and Australia

and New Zealand (FSANZ, 2016) as well as the Codex Alimentarius (Alimentarius, 2016).

Globally, Cd contamination is mainly localized in industrialized regions principally, Japan, China, Europe and North America, associated with heavy fossil fuel usage, industrial products and the heavy use of phosphate fertilisers (ATSDR, 2012). The Soil Geochemical Atlas of Ireland indicates a large area in north Leinster (Figure 1.1) with greater natural concentrations of Cd due to the underlying geology (Fay *et al.*, 2007). This area contains over 50% of Irish horticultural production. A survey indicated that a large proportion of potato, parsnip, and leafy vegetables have Cd concentrations close to or exceeding the Maximum Limits (MLs) (Teagasc, unpublished results). Produce exceeding the Cd MLs will be removed from the market with severe financial consequences for primary producers. Currently, no Cd mitigation advice is available to Irish producers.

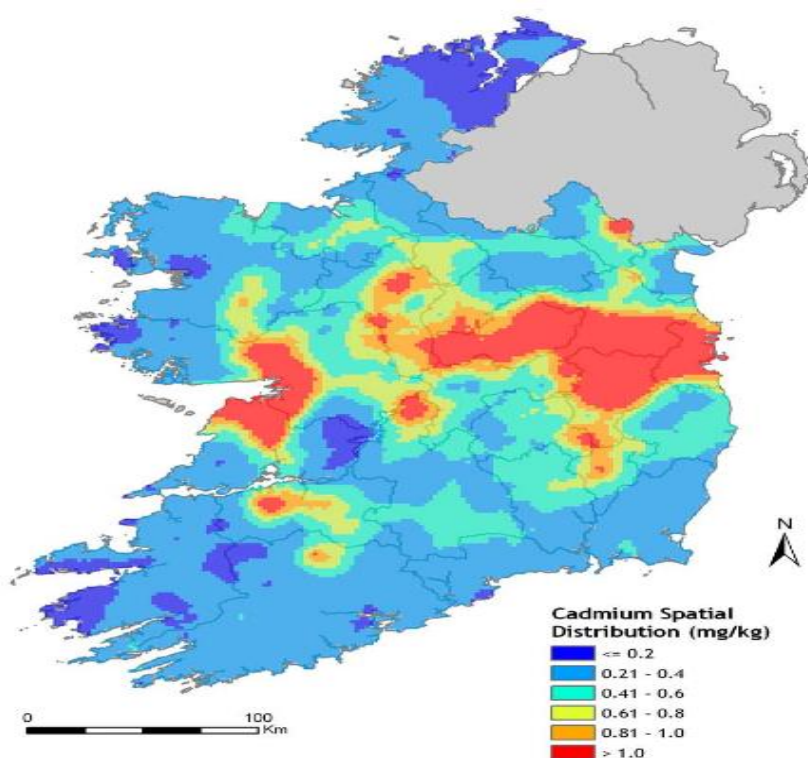


Figure 1.1 Spatial distribution map for Cd in Irish soils (Fay *et al.* 2007).

Research into strategies to reduce Cd availability in soil, and its uptake by plant roots is extensive. Agronomic methods such as phytoextraction and soil amendments have been widely tested (Ueno *et al.*, 2009). However, the practical applicability of these approaches is currently limited, due to the fact that they tend to be slow, expensive and only partly effective in some soils (Ueno *et al.*, 2009). Other approaches for dealing

with Cd-rich soils, such as avoidance, may be practiced but may not always be practicable in land-limited situations. Breeding genotypes with less Cd in the edible part of the plant is another potential avenue for dealing with soils in which Cd has become a problem.

Breeding for genotypes accumulating less Cd may also be complicated because of the similarity of Cd's physical and chemical properties to those of zinc (Zn). Zinc is an essential micronutrient for human health. Zinc malnutrition has resulted in serious health problems in humans, including impaired function of brain, immune and reproductive system and energy metabolism, stunted growth, diarrhoea and pneumonia, especially in children (White & Broadley, 2009, 2011; Sperotto, 2013; White *et al.*, 2017). Zn malnutrition can be addressed through food diversification, food fortification or mineral supplementation. However, it is not always successful in regions where food diversification and supplementation are not easily implemented (White & Broadley, 2011; White *et al.*, 2017). An alternative intervention, termed biofortification, is an important strategy by which edible portions are enriched with Zn while the plants are growing (White & Broadley, 2011; Kromann *et al.*, 2017; White *et al.*, 2017). Therefore, care must be taken not to reduce Zn concentration in the edible plant parts while genetics and agronomic interventions are being applied to reduce Cd concentration. An understanding of the physiological and genetic mechanisms by which both Cd and Zn accumulate in plant parts provides a framework for the development of genotypes accumulating less Cd and more Zn simultaneously in the same breeding programme. Within this context, the research described in this thesis addressed both Cd and Zn simultaneously.

Chapter 2: Literature Review

2.1. Cd and Zn uptake, transport and distribution

Cd and Zn accumulation in edible plant parts, including tubers, is not a single-factor process (McLaughlin *et al.*, 1994, 1997; Di Toppi & Gabbrielli, 1999; Broadley *et al.*, 2007; Gallego *et al.*, 2012; Sheoran *et al.*, 2016). Instead, it is a complex and dynamic process which is influenced by multiple factors, including plant species, cation type, cation concentration and the availability of the cations in the rhizosphere. Circumstances which affect these factors could also contribute to differences in uptake, transport and distribution of the two elements to edible portions (Gallego *et al.*, 2012)

Plants take up Cd and Zn from the soil through the roots and transport them to the above-ground plant parts via the xylem pathway. In the above-ground parts, Cd and Zn are stored in the shoot and/or redistributed to organs including seeds, fruits, roots and tubers (Reid *et al.*, 2003; Gallego *et al.*, 2012; Olsen & Palmgren, 2014; Yamaji & Ma, 2014).

2.1.1. Factors affecting soil bioavailability of Cd and Zn

The degree to which plants accumulate Cd and Zn depends on soil concentration of these elements, and soil- and plant-related factors which modulate bioavailability of the elements in the rhizosphere (Kidd *et al.*, 2009). Bioavailability is defined as “the fraction of the total element content of the soil that can interact with a biological target” (Kidd *et al.*, 2009).

Cd can exist in both solid and liquid phases in the soil (Clemens, 2006; Kidd *et al.*, 2009; Gallego *et al.*, 2012). However, plants take up Cd in the free cation (Cd^{2+}) form and, to some degree, in the Cd-chelate form from the soil solution. Therefore, for uptake by roots, Cd must be in the free ion form (Cd^{2+}) or in slightly soluble forms such as Cd^{2+} complexed with organic ligands (Cd^{2+} -organic acids, Cd^{2+} -humate, etc.) or adsorbed onto inorganic and organic soil particles at ion-exchange sites (Clemens, 2006; Kidd *et al.*, 2009; Gallego *et al.*, 2012).

Similarly, Zn can be found as water-soluble (including Zn^{2+} and soluble soil fractions), adsorbed and exchangeable Zn in the colloidal fractions (i.e. associated with clay particles, humic compounds and Al and Fe hydroxides) and insoluble Zn complexes and minerals (Broadley *et al.*, 2007). Plants mainly take up Zn from soil solution in the free ion (Zn^{2+}) form (Broadley *et al.*, 2007).

Phytoavailable Cd and Zn in the soil are determined by a combination of soil- and plant-associated factors (McLaughlin *et al.*, 1994, 1997; Di Toppi & Gabbrielli, 1999; Broadley *et al.*, 2007; Sheoran *et al.*, 2016). Soil-associated factors, such as chemical (e.g. pH, organic matter content, cation exchange capacity) and physical properties (texture, structure and bulk density), determine the amounts of exchangeable and water-soluble Cd and Zn in the soil (McLaughlin *et al.*, 1994, 1997; Di Toppi & Gabbrielli, 1999; Broadley *et al.*, 2007; Sheoran *et al.*, 2016).

Among the soil factors, soil pH is the major factor influencing Cd and Zn bioavailability in the rhizosphere (Cakmak, 2008; Kidd *et al.*, 2009; Gallego *et al.*, 2012; Sheoran *et al.*, 2016). Phytoavailable Cd and Zn increase with a decrease in soil pH. A decrease in soil pH results in more H⁺ ions which have strong attraction for negatively-charged surfaces of soil particles and which can displace other cations from organic ligands and complexes (McLaughlin *et al.*, 1994, 1997; Di Toppi & Gabbrielli, 1999; Clemens, 2006; Kirkham, 2006; Broadley *et al.*, 2007; Cakmak, 2008; Kidd *et al.*, 2009; Gallego *et al.*, 2012; Sheoran *et al.*, 2016).

Soil moisture is another important soil factor, providing as it does a suitable medium for an adequate diffusion of cations, including Zn and Cd, to plant roots. Plants grown in soils with greater moisture content accumulate an more Cd and Zn compared to those grown under moisture-deficient conditions (Cakmak, 2008; Sheoran *et al.*, 2016).

Soil organic matter also influences bioavailability through retention of transition cations. Previous studies reported that soils with a greater organic matter (OM) content provide more diethylenetriaminepentaacetic acid (DTPA)-extractable Zn than can be obtained from a lower OM soil (Cakmak, 2008). However, Nigam *et al.* (2001) conducted an experiment by applying low molecular-weight organic acids (malic, citric and aspartic acid) and found that Cd concentration was increased in all tissues of maize. The authors suggested that increased Cd concentration in the plant tissues may be associated with an increase in mobile organically-bound Cd in the soil as result of the low molecular weight organic acids (LMWOAs). Similar results have been reported in other studies (Kirkham, 2006; Montiel-Rozas *et al.*, 2016). It is generally accepted that high molecular weight organic acids (HMWOAs) reduce bioavailability and mobility of heavy metal. On the other hand, soils with an increased concentration of LMWOA exhibit greater bioavailability and mobility of elements including Cd and Zn. Therefore,

composition and types of soil OM determine the behaviour of these cations bioavailability in the soil (Kidd *et al.*, 2009).

In addition to soil factors, plant-soil interactions and plant-associated microorganisms such as mycorrhizae and root-colonizing bacteria also affect soil Cd and Zn bioavailability (McLaughlin *et al.*, 1994, 1997; Clemens, 2006; Broadley *et al.*, 2007; Cakmak, 2008; Kidd *et al.*, 2009; Sheoran *et al.*, 2016). Plant roots influence element availability in the rhizosphere through acidification by pumping out H⁺ ions when taking up cations and actively secreting organic acids which chelate heavy metals in the soil. Examples of organic acids released into the soil as result of iron deficiency are phytosiderophores or other organic acids like citrate and oxalate (Clemens, 2006; Kidd *et al.*, 2009; Montiel-Rozas *et al.*, 2016; Sheoran *et al.*, 2016).

Organic acids change the behaviour of the rhizosphere and enhance the uptake of Fe and Zn (Ishimaru *et al.*, 2012). For example, siderophores, which are high-affinity iron-chelating compounds, secreted by plant roots during Fe and Zn deficiency. The release of siderophore to the soil changes behaviour in the rhizosphere and increase the uptake of Fe and Zn from the soil solution. Because of the similarity between Cd and both Fe and Zn, changes in the rhizosphere may also increase the uptake of Cd (Ishimaru *et al.*, 2012).

Root exudates, such as amino acids, low molecular weight carboxylic acids, sugars and simple phenolic acids and flavonoid-type phenolics, secretions (ectoenzyme and polymeric carbohydrates), plant mucilages (carbohydrate and plant debris) and root lysates, are all important in changing the pH, redox potential, organic ligand concentrations and microbial biomass of the rhizosphere (Kidd *et al.*, 2009; Gallego *et al.*, 2012; Sheoran *et al.*, 2016). However, these rhizosphere changes depend on the plant species (due to differences in type, amount and timing of exudate production and root architecture), soil types and properties, and cation types and concentrations (Kidd *et al.*, 2009; Gallego *et al.*, 2012; Montiel-Rozas *et al.*, 2016; Sheoran *et al.*, 2016). Montiel-Rozas *et al.* (2016) assessed the effects of the amount and types of LMWOA production in response to Cd, Cu and Zn treatments at different concentrations and with respect to plant species (*Poa annua*, *Medicago polymorpha* and *Malva sylvestris*). The authors reported that the amount and types of exudates depended strongly on plant species and cation concentrations. In general, factors affecting soil pH and

mineralization (ionization and complex formation) controlled the availability of Cd and Zn in the soil solution.

2.1.2. Cd and Zn uptake

Once bioavailable in the soil solutions, Cd and Zn, are taken up by the roots and transported into the plant through the apoplastic pathway (Muschitz *et al.*, 2009; Gallego *et al.*, 2012; Sheoran *et al.*, 2016). The apoplast is the space outside the plasma membrane, comprising the cell wall continuum and intercellular spaces, which allows the movement of ions and solutes through it. The process does not require energy and operates in the non-living part of the tissue. The movement is, therefore, not affected by the metabolic state of the root or on plasma membrane proteins (Muschitz *et al.*, 2009; Gallego *et al.*, 2012; Sheoran *et al.*, 2016). However, this pathway is restricted by a band of cell wall material (the Casparian strip) deposited in the circumferential and longitudinal walls of the endodermis, which is chemically different from the rest of the cell wall; the cell wall is made of cellulose and lignin but without suberin whereas the Casparian strip is made of suberin and some lignin. In addition to the Casparian strip, the apoplastic sites are negatively charged, due mainly to the presence of carboxyl groups. As a result, Cd and Zn are also influenced by competition with other cations, including calcium, magnesium and potassium, for these apoplastic sites (Broadley *et al.*, 2007; Marques *et al.*, 2007; Muschitz *et al.*, 2009; Lux *et al.*, 2010; Haydon *et al.*, 2012; Samardjieva *et al.*, 2015).

Given the importance of solutes and ions to the cells and the presence of Casparian strips across the apoplast, ions and solutes are forced to enter the cell by crossing the plasma membrane of the cell. The plasma membrane is characterized as a selectively permeable outer cover of the living cell, separating the living cell from the outside environment, intercellular spaces and cell wall. It is a phospholipid bilayer, where the phosphate head is polar, the fatty acid tails are non-polar, and proteins are embedded in the membrane. These proteins regulate entry and exit of solutes and ions by acting as pumps, channels, receptors, enzymes and structural components (Clemens, 2006; Broadley *et al.*, 2007; Sasaki *et al.*, 2012).

With the aid of membrane-transporter proteins, ions and solutes enter the cells, and are transported from cell to cell through plasmodesmata connections, operating in the living cell part of the root and highly influenced by the metabolic state and energy demand of

the cell. This process of ion and solute transport is called the symplastic pathway. Therefore, the first step for symplastic movement of ions and solutes is to cross the plasma membrane. The plasma membrane is selectively permeable, preventing the entry of toxic substances into the cell. Cd is non-essential to plant cells and it is believed that plants do not have Cd-specific membrane transporters (Clemens, 2006; Broadley *et al.*, 2007; Sasaki *et al.*, 2012). Instead, Cd²⁺ can cross the plasma membrane via other cation transporters, such as Zn²⁺ and Fe²⁺ transporters because of the similar chemical properties exhibited by these cations (Clemens, 2006; Broadley *et al.*, 2007; Sasaki *et al.*, 2012). Therefore, it is generally accepted that the uptake and transport of Cd²⁺, via the symplastic pathway is associated with the specific ion transporters of Zn²⁺ and Fe²⁺.

Studies have shown that the ZIP (Zinc-regulated transporter/Iron-regulated transporter-like protein) family of plasma membrane transporters (Connolly *et al.*, 2002; Vert *et al.*, 2002; Nakanishi *et al.*, 2006) mediate the uptake of Cd, Zn, and Fe. The ZIP family transporters, such as AtIRT1 and AtIRT2, mediate the root uptake of Cd²⁺, Fe²⁺, Mn²⁺ and Zn²⁺ in *A. thaliana* (Römheld & Marschner, 1986; Connolly *et al.*, 2002; Vert *et al.*, 2002; Nakanishi *et al.*, 2006). Other ZIP family members, OsIRT1 and OsIRT2, are responsible for the uptake of Cd, Fe and Zn in rice (Nakanishi *et al.*, 2006; Ishimaru *et al.*, 2012; Yoneyama *et al.*, 2015). Expression of the two corresponding genes are induced by iron deficiency (Nakanishi *et al.*, 2006).

Members of the Nramp (Natural resistance associated macrophage protein) family are also involved in root uptake of Cd (Thomine *et al.*, 2000; Cailliatte *et al.*, 2009; Takahashi *et al.*, 2011; Sasaki *et al.*, 2012; Wu *et al.*, 2016). Among the Nramp family, AtNramp 1, 3, and 4 in *Arabidopsis thaliana* (Thomine *et al.*, 2000), OsNramp1 and OsNRAMP5 in rice (Cailliatte *et al.*, 2009; Takahashi *et al.*, 2011; Sasaki *et al.*, 2012) and HvNramp5 in barley (Wu *et al.*, 2016) are involved in root uptake of Cd. OsNramp1 shows transport activity for Fe and Cd in yeast and is proposed to be involved in Cd transport in rice (Takahashi *et al.*, 2011). *OsNramp5* is constitutively expressed in the rice root at all stages of growth and enhances root uptake and transport of Cd. The knockout of this gene resulted in total loss of Cd uptake but showed no response to Fe-deficiency (Takahashi *et al.*, 2011; Ishikawa *et al.*, 2012). OsNramp5 functions as a major transporter responsible for root Mn and Cd uptake (Sasaki *et al.*, 2012). Recently its close homologue in barley, HvNramp5, was identified and shown to be involved in the uptake of both Cd and Mn, but not of Fe, by barley roots (Wu *et al.*, 2016). In addition to these, Cd²⁺ can enter plant root cells via other cation channels, such as

depolarization-activated calcium channels (DACC), hyperpolarisation-activated calcium channels (HACC), and voltage-insensitive cation channels (VICC) (Lux *et al.*, 2010).

2.1.3. Fate of Cd and Zn inside plants

Once the cations, including Cd^{2+} and Zn^{2+} , are inside the plant root, their possible fates include immobilization in the apoplast (metal binding to the cell wall and intercellular spaces), chelation in the cytoplasm, compartmentalisation in the vacuole, loading to the xylem and translocation to the shoot and/or efflux back to the soil solution (Gallego *et al.*, 2012; Haydon *et al.*, 2012; Yoneyama *et al.*, 2015).

2.1.3.1. Immobilization in the apoplast pathway

The cell wall is the first barrier to the entry of cations, including Cd^{2+} and Zn^{2+} , into the plant cells. As Cd^{2+} and Zn^{2+} pass through the cell wall, they can be bound to the cell wall (Leita *et al.*, 1996; Küpper *et al.*, 2000; Muschitz *et al.*, 2009; Samardjieva *et al.*, 2015; Yoneyama *et al.*, 2015; Gao *et al.*, 2016). The cell wall is characterized by negatively-charged sites, as a result of pectic acids and histidyl groups that can bind cations including Cd^{2+} and Zn^{2+} (Frey *et al.*, 2000; Marques *et al.*, 2007; Samardjieva *et al.*, 2015; Yoneyama *et al.*, 2015; Gao *et al.*, 2016).

Intercellular distribution analysis of Cd in bush bean showed that most of the Cd^{2+} was bound to the pectic sites and histidyl groups of the cell wall in roots and leaves (Leita *et al.*, 1996). Similar studies in *Arabidopsis halleri* also showed that the majority of Cd^{2+} and Zn^{2+} was bound to the cell wall of the rhizodermis (root epidermis), mainly due to precipitation as Zn/Cd phosphates. In leaves, Cd^{2+} and Zn^{2+} were sequestered in the trichomes, and the concentrations of Cd^{2+} and Zn^{2+} in the mesophyll cells increased markedly in response to increasing Zn^{2+} and Cd^{2+} concentrations (Küpper *et al.*, 2000). Studies suggest that phosphorus played a significant role in blocking entry of Cd^{2+} or an elevated level of Zn^{2+} by binding these elements at the cell wall and in intercellular spaces (Küpper *et al.*, 2000; Lux *et al.*, 2010).

Cd^{2+} and Zn^{2+} can be deposited in the intercellular spaces, the space between the cell wall and the plasma membrane (Di Toppi & Gabbrielli, 1999; Frey *et al.*, 2000; Küpper *et al.*, 2000; Marques *et al.*, 2007; Gallego *et al.*, 2012; Samardjieva *et al.*, 2015). In the intercellular space, there are deposits of carbohydrates such as hemicellulose, mucilage

and callose. In these deposits, Cd^{2+} and Zn^{2+} can be precipitated (Di Toppi & Gabbrielli, 1999; Frey *et al.*, 2000; Küpper *et al.*, 2000; Marques *et al.*, 2007; Gallego *et al.*, 2012; Samardjieva *et al.*, 2015). Subcellular distribution analysis of Zn in solanaceous species, namely *Solanum nigrum* and *Solanum lycopersicum*, showed that Zn is mainly deposited in the intercellular spaces and on the cell wall (Marques *et al.*, 2007; Muschitz *et al.*, 2009; Samardjieva *et al.*, 2015). Furthermore, studies showed that enhanced pectin and hemicellulose contents in the cell wall, induced by exogenous application of nitric oxide, increased the cell wall deposition of Cd^{2+} and a decrease in Cd^{2+} concentration in other cellular components of rice (Gao *et al.*, 2016). Therefore, apoplast compartments (cell wall and intercellular spaces) play a significant role in reducing entry of Cd^{2+} and excess Zn^{2+} into the cytosol.

2.1.3.2. Symplastic sequestration

The entry of Cd^{2+} into the cytosol interferes with cellular activities and causes dysfunction of the plant (Di Toppi & Gabbrielli, 1999). Due to the high affinity of Cd^{2+} for sulphydryl groups, its entry into the cytosol is easily recognized by sulphur-containing metabolites, and activates S-containing ligands such as glutathione (GSH) and its derivatives, the phytochelatins (PCs) (Di Toppi & Gabbrielli, 1999). The phytochelatins are characterized as small Cd-binding peptides and are produced (in response to Cd^{2+}) from glutathione by the activity of PC synthase (Di Toppi & Gabbrielli, 1999; Gallego *et al.*, 2012). The phytochelatins have a general structure (g-Glu-Cys) *n*-Gly, where *n* is the number of repetitions of the unit g-Glu-Cys, which ranges from 2 to 11 and thereby form variants of PC (PC2 to PC11). Glutamate, cysteine, and glycine are the basic components of PC and glutathione is the key intermediate for PCs. These variants of PCs can form various complexes with Cd^{2+} in the cytoplasm (Di Toppi & Gabbrielli, 1999). These PC-Cd complexes (GSH-Cd conjugates) prevent cell damage by reducing circulation of the free ions of Cd (Cd^{2+}) in the cytosol (Di Toppi & Gabbrielli, 1999; Gallego *et al.*, 2012). However, these complexes still impede the biological processes of the cell, so plant cells avoid this disturbance by exporting them into the vacuoles. The plant vacuoles are known for storage of incompatible compounds due to their occupation of a large percentage of the cell volume and the limited metabolic activities (Di Toppi & Gabbrielli, 1999; Gallego *et al.*, 2012).

The transport of these PC-Cd complexes into the vacuole is mediated by transporter proteins located in the tonoplast of the cell (Song *et al.*, 2010; Park *et al.*, 2012; Brunetti *et al.*, 2015). Studies showed that the transport of PC-Cd complexes is mediated by ATP-binding cassette (ABC) transporters (Salt & Rauser, 1995; Song *et al.*, 2010; Park *et al.*, 2012; Brunetti *et al.*, 2015). The phytochelatin transporters, AtABCC1 and AtABCC2, have been identified as transporters of PC-Cd complexes into the vacuoles (Park *et al.*, 2012). In line with this, a recent study by Brunetti *et al.* (2015) reported the involvement of AtABCC3 in the vacuolar transport of PC-Cd complexes in coordination with AtABCC1 and AtABCC2 in *A. thaliana*. Cd²⁺ can also be transported directly to vacuoles by the vacuolar Ca²⁺/H⁺ antiporters (Clemens *et al.*, 2001). In rice, the tonoplast-localized transporter, OsHMA3, is involved in the transport of PC-Cd complexes to the vacuoles (Ueno *et al.*, 2010).

In the vacuoles, these complexes form more stable high molecular-weight complexes via sulphide bonds (Salt & Rauser, 1995). The transport to and formation of high molecular-weight complexes of PC-Cd in the vacuoles is an example of compartmentalisation (Salt & Rauser, 1995; Song *et al.*, 2010; Park *et al.*, 2012; Brunetti *et al.*, 2015). Studies have shown that the amount of high-molecular-weight Cd-ligands increases with Cd²⁺ concentration (Montiel-Rozas *et al.*, 2016).

With respect to elevated Zn²⁺ in the cytoplasm, plants avoid interference with cellular activities by exporting Zn²⁺ into the vacuoles (Broadley *et al.*, 2007). In the vacuoles, Zn²⁺ is predominantly complexed with nicotianamine (NA). Nicotianamine is a low-molecular-weight transition metal chelator molecule with a high binding affinity for Zn²⁺. It is synthesized enzymatically from three molecules of *S*-adenosyl methionine by nicotianamine synthases (NASs) (Broadley *et al.*, 2007; Haydon *et al.*, 2012; Yoneyama *et al.*, 2015).

Transporters, zinc-induced facilitator (ZIF), cation diffusion facilitator (CDF) family, including metal tolerance protein 1 (MTP1) and metal tolerance protein 3 (MTP3), and Mg²⁺/H⁺ antiporter (MHX) are involved in the transport of Zn²⁺ from the cytosol into the vacuoles via a H⁺/Zn²⁺ antiporter mechanism (Broadley *et al.*, 2007; Haydon *et al.*, 2012; Yoneyama *et al.*, 2015).

The entry of Cd²⁺ into the cytosol interferes with cellular activities and causes dysfunction of the plant (Di Toppi & Gabbrielli, 1999). Due to the high affinity of Cd²⁺

for sulphydryl groups, its entry into the cytosol is easily recognized by sulphur-containing metabolites, and activates S-containing ligands such as glutathione (GSH) and its derivatives, the phytochelatins (PCs) (Di Toppi & Gabbrielli, 1999). The phytochelatins are characterized as small Cd-binding peptides and are produced (in response to Cd^{2+}) from glutathione by the activity of PC synthase (Di Toppi & Gabbrielli, 1999; Gallego *et al.*, 2012). The phytochelatins have a general structure (g-Glu-Cys) n -Gly, where n is the number of repetitions of the unit g-Glu-Cys, which ranges from 2 to 11 and thereby form variants of PC (PC2 to PC11). Glutamate, cysteine, and glycine are the basic components of PC and glutathione is the key intermediate for PCs. These variants of PCs can form various complexes with Cd^{2+} in the cytoplasm (Di Toppi & Gabbrielli, 1999). These PC-Cd complexes (GSH-Cd conjugates) prevent cell damage by reducing circulation of the free ions of Cd (Cd^{2+}) in the cytosol (Di Toppi & Gabbrielli, 1999; Gallego *et al.*, 2012). However, these complexes still impede the biological processes of the cell, so plant cells avoid this disturbance by exporting them into the vacuoles. The plant vacuoles are known for storage of incompatible compounds due to their occupation of a large percentage of the cell volume and the limited metabolic activities (Di Toppi & Gabbrielli, 1999; Gallego *et al.*, 2012).

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Brunetti *et al.*, 2015). Studies have shown that the amount of high-molecular-weight Cd-ligands increases with Cd²⁺ concentration (Montiel-Rozas *et al.*, 2016).

With respect to elevated Zn²⁺ in the cytoplasm, plants avoid interference with cellular activities by exporting Zn²⁺ into the vacuoles (Broadley *et al.*, 2007). In the vacuoles, Zn²⁺ is predominantly complexed with nicotianamine (NA). Nicotianamine is a low-molecular-weight transition metal chelator molecule with a high binding affinity for Zn²⁺. It is synthesized enzymatically from three molecules of *S*-adenosyl methionine by nicotianamine synthases (NASs) (Broadley *et al.*, 2007; Haydon *et al.*, 2012; Yoneyama *et al.*, 2015).

Transporters, zinc-induced facilitator (ZIF), cation diffusion facilitator (CDF) family, including metal tolerance protein 1 (MTP1) and metal tolerance protein 3 (MTP3), and Mg²⁺/H⁺ antiporter (MHX) are involved in the transport of Zn²⁺ from the cytosol into the vacuoles via a H⁺/Zn²⁺ antiporter mechanism (Broadley *et al.*, 2007; Haydon *et al.*, 2012; Yoneyama *et al.*, 2015).

2.1.3.3. Xylem loading of Cd and Zn

Depending on plant species, part of the Cd²⁺ and Zn²⁺ content is sequestered in the root apoplast (cell wall and intercellular spaces) and symplast (vacuoles of the cell) while the remainder is loaded into the xylem, the dead vascular tissue leading from the root to the shoot (Broadley *et al.*, 2007; White & Broadley, 2009, 2011; Gallego *et al.*, 2012; Olsen & Palmgren, 2014).

Loading of Cd²⁺ and Zn²⁺ from the symplast to the xylem is mediated by transporter proteins (Olsen and Palmgren, 2014). Heavy metal transport protein family P1B-ATPase (HMAs) homologues, such as HMA2 and HMA4, are involved in loading Cd²⁺ and Zn²⁺ from the symplast into the xylem (Broadley *et al.*, 2007; White & Broadley, 2009, 2011; Gallego *et al.*, 2012; Olsen & Palmgren, 2014). AtHMA2 and AtHMA4 regulate Zn homeostasis and Cd detoxification and xylem loading in *A. thaliana* (Verret *et al.*, 2004; Wong & Cobbett, 2009). Similarly, AhHMA4 (a homologue of AtHMA4) plays a significant role in translocation of Cd²⁺ and Zn²⁺ from root to shoot in *A. halleri* (Hanikenne *et al.*, 2008). A homologue of AtHMA2, OsHMA2 mediates the transport of Cd²⁺ and Zn²⁺ into the xylem of rice (Broadley *et al.*, 2007; White & Broadley, 2009; Olsen & Palmgren, 2014; Yoneyama *et al.*, 2015).

Once Cd and Zn are loaded into the xylem, they are translocated to the shoot via the xylem vessels. The xylem vessel is filled with xylem sap, and Cd and Zn are transported along with this xylem sap. The xylem sap is slightly acidic (pH 5.5-6) (Olsen & Palmgren, 2014; Yoneyama *et al.*, 2015). Earlier studies suggested that Cd²⁺ in the xylem sap could form complexes with compounds such as thiol ligands, organic acids and amino acids (Salt *et al.*, 1995; Welch & Norvell, 1999; Gong *et al.*, 2003). However, recent studies showed that Cd is predominantly found in the free ionic form in the xylem sap (Kato *et al.*, 2010; Hazama *et al.*, 2015; Yoneyama *et al.*, 2015). Examination of Cd-binding compounds in the xylem sap of castor bean revealed that only a minor fraction of the xylem sap Cd (less than 10%) was bound to certain thiol compounds, likely GSH. The majority of the Cd was in the free ion form (Hazama *et al.*, 2015). Similarly, studies also reported that Zn was predominantly found in the free ionic form along with minor fractions complexed with organic acids, histidine or NA in the xylem sap (Olsen & Palmgren, 2014; Hazama *et al.*, 2015; Yoneyama *et al.*, 2015).

2.1.3.4. Xylem unloading of Cd and Zn

Cd and Zn in the xylem can have three fates: (1) direct transport to the tuber, fruit or grain via the xylem; (2) continued root supply to the xylem and transfer to the phloem in the stem then transport to the tuber, fruit or grain i.e. xylem-to-phloem transfer in the stems; or (3) transport to the leaves and stems and storage in the stems and leaves, then remobilization to the sinks (tuber, root, grain or fruit) during the late growth stages (Hart *et al.*, 1998; Dunbar *et al.*, 2003; Reid *et al.*, 2003; Tanaka *et al.*, 2007; Rodda *et al.*, 2011; Huang *et al.*, 2015; Xin *et al.*, 2015, 2017).

Xylem-mediated transport of ions and solutes is directed mainly to the organs exhibiting the greatest transpiration, such as the leaves, but not to the organs which act as sinks (Marschner, 1995; Hart *et al.*, 1998; Dunbar *et al.*, 2003; Tanaka *et al.*, 2007; Rodda *et al.*, 2011; Huang *et al.*, 2015). Studies showed that Cd and Zn transported in the xylem mainly accumulated in the vegetative tissues (Hart *et al.*, 1998; Dunbar *et al.*, 2003; Reid *et al.*, 2003; Tanaka *et al.*, 2007; Rodda *et al.*, 2011; Xin *et al.*, 2015, 2017). Given that the rate of transpiration by tubers is very low and that there is no direct xylem-based connection between basal roots and tubers, the majority of Cd and Zn is transported to the tubers via the phloem pathway (Kratzke & Palta, 1985, 1986; Dunbar *et al.*, 2003; Reid *et al.*, 2003). Studies in other crops also showed similar results with Cd and Zn transport to the sinks (grains, fruits and tubers) being predominantly

via the phloem pathway (Reid *et al.*, 2003; Tanaka *et al.*, 2007; Yoneyama *et al.*, 2010, 2015; Rodda *et al.*, 2011; White & Broadley, 2011; Hazama *et al.*, 2015).

The phloem sap is characterized as being slightly alkaline (pH 8) and Cd in the phloem sap is bound to proteins, organic acids, phytochelatins and glutathione (Reid *et al.*, 2003; Tanaka *et al.*, 2007; Kato *et al.*, 2010; Yoneyama *et al.*, 2010, 2015; Hazama *et al.*, 2015), whereas Zn in the phloem sap is predominantly bound to nicotianamine (NA) and to some degree with phytochelatins, glutathione, phytosiderophores and organic acids (Rodda *et al.*, 2011; White & Broadley, 2011; Olsen & Palmgren, 2014; Hazama *et al.*, 2015; Yoneyama *et al.*, 2015).

Previous studies showed that continued root to xylem-phloem [transfer in the stem was the main source of Cd (Sankaran & Ebbs, 2008; Fujimaki *et al.*, 2010; Yoneyama *et al.*, 2010; Yamaji & Ma, 2014; Xin *et al.*, 2015) to the developing sinks. Image analysis of Cd isotope distribution showed that xylem-to-phloem transfer in the stem is the source of Cd for phloem transport to the grains of rice (Fujimaki *et al.*, 2010; Yamaguchi *et al.*, 2012; Yamaji & Ma, 2014). Furthermore, OsLCT1, identified as a plasma membrane-localized low-affinity cation transporter, may participate in Cd transport to the grains (Uraguchi *et al.*, 2011, 2014). According to the authors, OsLCT1 was highly expressed in node I while suppression resulted in a decrease in Cd transport to the grains.

Studies also showed that xylem-to-phloem transfer in the stem plays a significant role in the partitioning of Zn to the sinks (Garnett & Graham, 2005; Jiang *et al.*, 2008; Waters & Grusak, 2008; Yoneyama *et al.*, 2010; Yamaguchi *et al.*, 2012; Sankaran & Grusak, 2014; Yamaji & Ma, 2014). It is generally accepted that the nodes are the hub for Cd and Zn distribution, acting as “control traffic centre” in partitioning Cd and Zn from the stem to the different organs (Yamaji & Ma, 2014). On the other hand, numerous studies have shown that remobilization of Cd and Zn from vegetative tissues is the main source of Cd and Zn to the grains or fruits. Most studies have suggested that both continued root to xylem-phloem transfers in the stem and remobilization from vegetative tissues are the main sources of Cd and Zn to the developing grains or fruit (Garnett & Graham, 2005; Jiang *et al.*, 2008; Waters & Grusak, 2008; Yoneyama *et al.*, 2010; Yamaguchi *et al.*, 2012; Sankaran & Grusak, 2014; Yamaji & Ma, 2014). It is generally accepted that the nodes are the hub for Cd and Zn distribution, acting as

“control traffic centre” in partitioning Cd and Zn from the stem to the different organs (Yamaji & Ma, 2014).

2.2. Potato

Potato (*Solanum tuberosum* L.) is the fourth largest food crop worldwide, after maize, rice and wheat with an estimated annual production of 382 million tonnes (FAOSTAT 2014). It occupies a wide eco-geographical range from temperate to tropical highlands. It is a unique crop that produces stolons (below-ground horizontal stems) which swell at the tips to form tubers (Potato Genome Sequencing Consortium 2011). The tubers are a globally important dietary source of carbohydrates, protein, antioxidants, vitamins e.g. vitamin C, thiamine, niacin, pyridixine, vitamin B6, riboflavin, folic acid, and minerals e.g. calcium, magnesium and phosphorus (Bradshaw *et al.*, 2006). In addition to their roles as sources of food and industrial starch, the tubers also act as a vegetative propagation system (Ferne & Willmitzer, 2001; Bradshaw *et al.*, 2006).

2.2.1. Genetics and taxonomy

Potato belongs to the Solanaceae family and the genus *Solanum* section Petota. The Solanaceae, which includes tomato, pepper, aubergine (eggplant), petunia and tobacco, is one of the most significant and diverse families of the kingdom Asterids. Potato has a basic chromosome number of 12 and a genome size of 844 mega base pair (Mbp), making it a medium-sized genome (Potato Genome Sequencing Consortium 2011). Potato has unique features, including both asexual and sexual reproductive systems; the production of gametes with unreduced chromosome number; the existence of different ploidy levels; and the presence of an endosperm dosage system that regulates interploidy/interspecific crosses (Carputo *et al.*, 2003; Carputo & Barone, 2005).

Taxonomical classification of both cultivated and wild potato species are very complex and differ among experts. The ploidy level of potato species has been one of the most important taxonomic characters to identify cultivated potato species. Recent classification of section Petota showed that potato and its relatives include four cultivated species and about 100 wild species. About 70% of the wild species are diploid ($2n=2x=24$), with the remainder being tetraploid ($2n=4x=48$) or pentaploid ($2n=5x=60$). The cultivated potatoes are predominantly tetraploid and highly heterozygous ($2n=4x=48$) (Cai *et al.*, 2012).

Biological features of potato, such as the formation of gametes with unreduced chromosome number ($2n$), endosperm balance number (EBN) incompatibility and self-incompatibility, contribute to the formation of sexual polyploidization, diverse genetic resources and complicated taxonomic classification (Carputo & Barone, 2005; Spooner, 2009).

Potato genotypes produce unreduced gametes as a result of meiotic anomalies, affecting either micro-or macrosporogenesis (Carputo *et al.*, 2003; Carputo & Barone, 2005). Unreduced gamete formation is the basis for sexual polyploidization events. The second important feature is the EBN, which influences the success of interploid and interspecific crosses. The EBN is a number varying from 1 to 4, expressing the effective ploidy of *Solanum* species (Carputo *et al.*, 2003; Carputo & Barone, 2005). Cultivated *S. tuberosum* is 4 EBN, whereas most of the wild species (either diploid or tetraploid) are 2 EBN. Normal fertilisation and seed development will happen if the maternal EBN is twice that of the paternal EBN (2:1 ratio) in the hybrid endosperm. The natural occurrence of unreduced gametes makes it possible that species with lower EBN can cross with species with larger EBN, for example a diploid species with 2 EBN and a tetraploid species with 4 EBN can produce a successful hybrid if the parent with lower ploidy produces $2n$ gametes (Carputo *et al.*, 2003; Carputo & Barone, 2005). Moreover, self-incompatibility is a common phenomenon for most wild and cultivated potatoes (Carputo *et al.*, 2003; Carputo & Barone, 2005; Spooner, 2009). The incompatibility is of a gametophytic multi-allelic nature, based on the occurrence of S alleles. This leads to outcrossing. In general, $2n$ gamete formations, EBN and self-incompatibility, together with geographical and ecological isolation, play a key role in maintaining complicated taxonomic classification, wide speciation and diverse genetic resources in potato (Carputo *et al.*, 2003; Carputo & Barone, 2005; Spooner, 2009).

2.2.2. Origin and domestication of potatoes

The centres of diversity for wild tuber-bearing *Solanum* species are located in the Americas, from the south-western United States to central Argentina and Chile (Hawkes, 1990; Spooner *et al.*, 2004). The three geographical areas of the American continent traditionally described as sources of wild potato are a) an equatorial area with short-day species (ser. *Andigena*); b) lowland areas of Chile and closely situated islands with long-day species (ser. *Tuberosa* and *Etuberosa*); and c) a lowland area of Uruguay (ser. *Commerisionianna*) (Hawkes, 1990; Spooner *et al.*, 2004).

According to Hijmans and Spooner (2001), wild potatoes occur in 16 American countries, but 88% of the wild species are from Argentina, Bolivia, Mexico and Peru. Most of the species are rare and narrowly endemic. Peru has the largest number of species followed by Bolivia. Species richness also occurs in northern Argentina, central Bolivia, central Ecuador and central Mexico. Peru has also rich in number of rare potato species (Hijmans & Spooner, 2001).

Potato was probably first domesticated from diploid wild species in the Lake Titicaca region of Peru and Bolivia between 10,000 and 7,000 years ago. The first cultivated diploid potatoes (*S. phureja* and *S. stenotomum*) are believed to be the progenitors for the tetraploid potato *S. tuberosum* subsp. *andigena*, which is a short-day-adapted Andean potato. It is also thought that the subspecies *tuberosum* crossed with wild species and produced longer-day potatoes with a more southerly distribution (Hawkes, 1990; Spooner *et al.*, 2004; Bradshaw *et al.*, 2006).

Potatoes are believed to have been introduced to Europe in the 1570s (Bradshaw *et al.*, 2006). However, there are no written records indicating when or how potato was introduced and domesticated in Europe and other regions. As a result, the origin of the European potato has been a subject of long debate, centring on a Chilean vs. an Andean origin. The Chilean origin hypothesis was suggested because of similarities between Chilean landraces of *Solanum tuberosum* ssp. *tuberosum* and modern European cultivars, with respect to two major traits, namely morphology and tuberization under long days. Alternatively, the Andean origin hypothesis proposed that these two major traits of European potato evolved rapidly from Andean landraces of *Solanum tuberosum* subsp. *andigena* to a Chilean-type potato through aggressive positive selection following importation to Europe, as a result of similar environmental conditions between the Chilean lowlands and Europe (Hancock, 2012).

Ames and Spooner (2008) settled the long-standing debate, using DNA from herbarium specimens. Analysis of a plastid DNA deletion marker from historical herbarium specimens suggested that Andean potato predominated in Europe during in the 1700's, but the Chilean potato was imported to Europe as early as 1811 and became dominant long before the occurrence of late blight epidemics in Ireland and the UK, after which Chilean potato was widely used in an attempt to find resistance.

2.2.3. Growth habit of potatoes

Potato is an annual C₃ herbaceous plant with wide genetic diversity. The wide genetic diversity of potato is also reflected in terms of growth habit, such as the number of branches and the pattern of branch development (Almekinders & Struik, 1996; Struik & Wiersema, 1999).

Potato has five major growth stages; tuber sprouting, vegetative growth, tuber initiation, tuber bulking and maturity (Struik and Wiersema, 1999). Tuber sprouts develop from the eyes of the tuber and enlarge to produce the stem of the potato. In the early stage of plant development, the mother tuber is the only energy source for growth. Following stem emergence, the stem develops the various elements of all the vegetative parts of the plants (leaves, branches, roots, and stolons). During vegetative growth, roots are also actively growing and providing nutrients and water. The root structure can be classified into basal root, stolon root, junction root and tuber root (Kratzke & Palta, 1985). The basal roots arise from the base of the main stem. They are relatively abundant compared to the other roots. The junction roots arise at the junction of stolons and main stems. The roots that arise from stolons (stolon roots) mainly emerge at the node of the stolons. The roots that arise directly from the base of the buds on the tuber are called tuber roots. The role of each root in water uptake and movement was examined using a water-soluble dye (Kratzke & Palta, 1985). The results showed that the basal and the junction roots do not appear to transport water to the tuber under field conditions. However, the stolon roots and the tuber roots transport water to the tuber under similar moisture conditions (Kratzke & Palta, 1985). Studies found that functional xylem connections do not exist between the tuber and basal roots. It is expected that water and ions are transported via the xylem to the shoot and then translocated to the tuber via the phloem pathway (Kratzke & Palta, 1985, 1986; Dunbar *et al.*, 2003; Reid *et al.*, 2003).

During the vegetative growth stage, the main stem produces leaf primordia. The numbers of leaf primordia are determined by the conditions during storage and pre-sprouting and the growth conditions after planting (Struik & Wiersema, 1999). The main stem then produces an inflorescence (a cyme), which either aborts during plant growth or develops fully. Following inflorescence initiation, vegetative growth is taken over by one of the axillary buds below the inflorescence, which develops into a secondary stem, and the secondary stem produces leaves and an inflorescence, and its

growth may be taken over by a third layer, etc. This sequence of growth results in different layers of branches which end with an inflorescence. The number of successive orders and patterns of branches strongly depends on the cultivar and, to some extent, on environmental conditions, age and type of planting materials as well as on agronomic management (Almekinders & Struik, 1996; Struik & Wiersema, 1999).

Based on the number and patterns of branching, cultivars are classified as determinate or indeterminate types (Almekinders & Struik, 1996; Struik & Wiersema, 1999). Determinate types tend to remain short, do not produce many successive orders of branches within one main stem and tend to exhibit a short life cycle. On the other hand, the indeterminate types may produce many different levels of branching, have a longer growing season and increased yield potential under suitable conditions (Almekinders & Struik, 1996; Struik & Wiersema, 1999). These conditions relate to the important agronomic trait of maturity, with early varieties exhibiting a determinate habit and late varieties tending towards a more indeterminate habit.

In addition to above-ground growth, the basal nodes of a stem produce a lateral shoot (stolon) that grows below the soil surface. The stolons are characterized by elongated internodes, hooked apical tips and diageotropic growth (Ferne & Willmitzer, 2001; Viola *et al.*, 2001). Potato tubers develop from the tip of the stolon. When environmental conditions are favourable for tuber initiation, the tips of stolon swell and form a tuber. Stolon-tuber transition depends on genetic and environmental factors. Environmental factors such as nitrogen doses, light intensity, photoperiod and the physiological age of the tuber affect the stolon-tuber transition. Limited nitrogen supply, short photoperiod and more light intensity promotes stolon-tuber transition (Ferne & Willmitzer, 2001; Viola *et al.*, 2001). Internal hormonal changes, including gibberellins, cytokinins, abscisic acid and others, also play a role in the stolon-to-tuber transition. Gibberellic acid is known to delay tuberization, whereas abscisic acid promotes tuber initiation. The stolon-tuber transition also results in massive physiological changes, especially the internal composition of carbohydrates and proteins. During stolon-tuber transition, the most abundant tuber proteins, including patatin and various proteinase inhibitors, play a role in tuber initiation. Changes in carbohydrate composition are also essential, studies showing that soluble carbohydrates, notably sucrose, are important for the induction of tuberization (Almekinders & Struik, 1996; Struik & Wiersema, 1999; Ferne & Willmitzer, 2001; Viola *et al.*, 2001).

The young developing tuber becomes a major sink for photoassimilate (Fernie & Willmitzer, 2001; Viola *et al.*, 2001). During tuber bulking, tuber cells expand with the accumulation of minerals and carbohydrates as well as water. Finally, tubers become the largest sink of the potato plant, storing massive amounts of carbohydrates (mainly starch) as well as significant amounts of protein. Furthermore, tubers decrease their general metabolic activity and, as such, behave as typical storage sinks (Almekinders & Struik, 1996; Struik & Wiersema, 1999; Fernie & Willmitzer, 2001; Viola *et al.*, 2001). The final stage of growth is maturity, when the haulm turns yellow and loses its leaves, so that photosynthesis gradually decreases.

2.2.4. Advances in potato breeding and genetics

Breeding activities in potato, like other crops, have mainly concentrated on morphology-based selection of the best cultivars/clones following rounds of sexual recombination (Jansky, 2011; Zhao *et al.*, 2013). The existence of broad genetic diversity in potato offers the opportunity to improve potato with respect to a wide range of important traits, including resistance to biotic and abiotic stress, yield, processing quality, nutritional value and adaptation to new environments (Bradshaw *et al.*, 2006; Jansky, 2011; Zhao *et al.*, 2013).

Phenotypic selection is an important method for the development of modern varieties with increased yield, more dry matter content, uniform tubers, rich in nutritional values and resistance/tolerance to biotic and abiotic stresses (Bradshaw *et al.*, 2006; Jansky, 2011; Zhao *et al.*, 2013). However, the process of breeding a new variety takes a long time (10-15 years), is expensive and somewhat inefficient (Hirsch *et al.*, 2016). Therefore, alternative approaches such as marker-assisted selection and/or genomic-assisted breeding can be used to accelerate phenotype-based potato breeding and/or to increase its efficiency (Dekkers, 2002; Ragoussis, 2006; Agarwal *et al.*, 2008; Collard & Mackill, 2008; Hirsch *et al.*, 2016).

2.2.5. Molecular plant breeding

In the 1980s, the advent of DNA-based molecular markers afforded plant geneticists and breeders the opportunity to both dissect the genetic basis of simple and complex traits using genetic mapping approaches, and to potentially use this information to accelerate plant breeding by using trait-linked markers in marker-assisted selection

(MAS)-based strategies (Dekkers, 2002; Ragoussis, 2006; Agarwal *et al.*, 2008; Collard & Mackill, 2008; Hirsch *et al.*, 2016).

Unlike the morphological and protein markers that preceded them, DNA markers (molecular markers) are not affected by the environment, tissue or developmental stages of the plants, and are thus more suited to routine use in both gene discovery and marker-assisted-selection strategies (Dekkers, 2002; Ragoussis, 2006; Agarwal *et al.*, 2008; Collard & Mackill, 2008; Hirsch *et al.*, 2016). In addition, DNA markers are abundant and possess a much greater allelic variation compared to morphological and biochemical markers. Polymorphism due to insertion/deletion (INDELs), single nucleotides polymorphisms (SNP), structural variants (SV) and copy number variants (CNV) can be detected using a range of molecular biology techniques (Collard & Mackill, 2008). Molecular markers, such as Restriction Fragment Length Polymorphism (RFLP), AFLP (Amplified Fragment Length Polymorphism) and Simple Sequence Repeats (SSR), have been widely used to localise genes/QTLs or genomic regions responsible for traits of interest to breeders (Dekkers, 2002; Ragoussis, 2006; Collard & Mackill, 2008; Agarwal *et al.*, 2008), and some of these markers have been used in MAS-based breeding strategies. Both the gene discovery and MAS applications are briefly reviewed in the next sections.

2.2.5.1. SNP Genotyping

Genotyping is the process of assignment of different variants in the genetic make-up of an individual by examining the individual's DNA sequence using biological assays and comparing it to another individual's sequence or a reference sequence (Dekkers, 2002; Ragoussis, 2006; Agarwal *et al.*, 2008; Collard & Mackill, 2008; Hirsch *et al.*, 2016). Numerous approaches and platforms have been utilised over the past several decades, and these are reviewed by Dekkers (2002), Ragoussis (2006), Agarwal *et al.* (2008), Collard and Mackill (2008) and Hirsch *et al.* (2016). Most recently, advances in high-throughput genotyping platform development have made single nucleotide polymorphism (SNP) markers the most commonly used system across almost all complex eukaryotes. SNPs are variations that occur at single nucleotides within the DNA sequence of the genome among members of a biological species (Dekkers, 2002; Ragoussis, 2006; Agarwal *et al.*, 2008; Collard & Mackill, 2008; Hirsch *et al.*, 2016). Numerous methods for SNP genotyping have been developed and applied in different biological areas based on the objective and design of the projects. The choice of the

SNP genotyping method depends on the number of samples to be genotyped and the density of SNP markers (Agarwal *et al.*, 2008; Collard & Mackill, 2008; Hirsch *et al.*, 2016).

There are numerous approaches for assaying SNPs, but the majority of SNP genotyping methods are based on one of the following: restriction endonuclease digestion, primer extension, hybridization and oligonucleotide ligation (Ragoussis, 2006; Collard & Mackill, 2008; Agarwal *et al.*, 2008; Hirsch *et al.*, 2016) followed by techniques such as electrophoresis, mass spectrometry, fluorescence analysis and chemiluminescence for detection of SNP variation (Ragoussis, 2006). Different approaches vary as much in scale as they do in approaches, i.e. some detect SNPs at individual loci per assay, whereas others may interrogate many thousands of loci simultaneously. The most comprehensive approach is based on resequencing entire genomes of target genotypes, but this is obviously an expensive process. Other approaches exist that can target thousands of loci in a relatively cost-effective manner. For the purposes of this thesis I will review only the approach adopted for this study.

The Illumina Infinium assay

The Infinium II assay (Illumina, www.illumina.com) uses a two-colour single base extension method for discrimination of allelic variants at each SNP locus combined with the BeadChip for assay detection. It uses a combination of biotin-labelled ddCTP and ddGTP, and 2, 4-dinitrophenol (DNP)-labelled ddATP and ddUTP (Ragoussis, 2006). As presented in Figure 2.1, genomic DNA samples undergo a whole-genome amplification (WGA) followed by hybridisation to locus-specific 50mers on the BeadChip. Then, the samples undergo a single-base extension and staining procedure. Single-base extension of the oligos on the BeadChip, using the captured DNA as a template, incorporates detectable labels on the BeadChip and determines the genotype call for the sample. Finally, the BeadChips are imaged using the Illumina iScan+. Unlike the Golden Gate assay that utilizes universal primers to amplify SNP-reactive DNA fragments, the Infinium assay relies on direct hybridization of genomic targets to array-bound sequences. The genotype calling is analysed using the Genome Studio software.

To facilitate the breeding efforts and our knowledge of potato, 600k genome-wide SNPs were discovered from six cultivated North America potato varieties using RNA-sequencing and expressed sequence tags (ESTs) (Hamilton *et al.*, 2011). Out of these,

69011 were high confidence SNPs suitable for development of an Illumina SNP genotyping array (Hamilton *et al.*, 2011). The information was utilised by the Solanaceae Coordinated Project (SolCAP) to develop what was called the Infinium 8303 Potato array as a genotyping resource for the potato genetics community (Felcher *et al.*, 2012).

Packages such as genotype calling (Voorrips *et al.*, 2011), incorporation of SNP dosages in map construction and quantitative trait loci (QTL) analysis (Hackett *et al.*, 2013, 2014, 2017) were developed. The SNPs from the Infinium 8303 Potato array have been applied to understand allelic variation for glycoalkaloid biosynthesis in wild and cultivated potatoes (Manrique-Carpintero *et al.*, 2014); to obtain a retrospective view of North American potatoes which represent a wide range of genetic resources (Hirsch *et al.*, 2013) ; to determine taxonomy and genetic differentiation between wild and cultivated potato (Hardigan *et al.*, 2015) ; to achieve genome-wide association mapping in European diploid and tetraploid potatoes (Stich *et al.*, 2013); and to carry out biparental linkage analysis (Hackett *et al.*, 2014; Massa *et al.*, 2015).

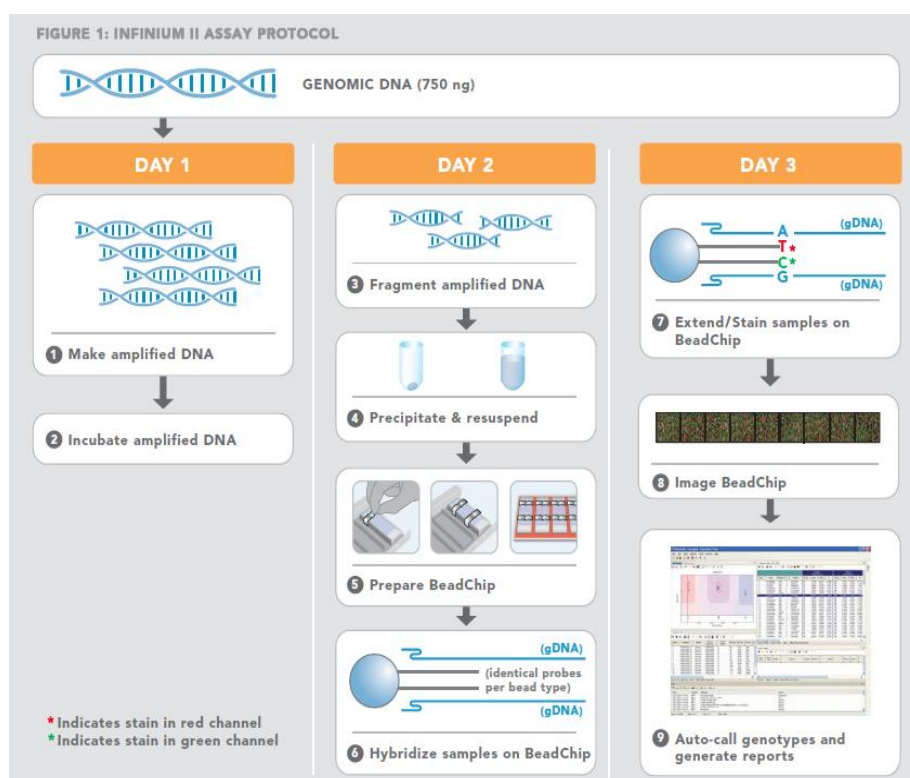


Figure 2.1 Diagrammatic representation of Infinium Array BeadChips.

(<http://dnatech.genomecenter.ucdavis.edu/infinium-assay/> retrieval date 20 March 2014).

2.2.5.2. Methods of Quantitative Trait Locus (QTL) detection

A quantitative trait locus (QTL) is a specific genome location that contributes to the quantitative variation of the trait of interest (Collard & Mackill, 2008). It is identified via statistical procedures that integrate genotypic and phenotypic data. QTL detection methods, such as linkage based QTL mapping and association mapping, have been used to identify genomic region(s) important for yield and other economically important traits in potatoes (Bradshaw *et al.*, 2004, 2008; Hackett *et al.*, 2013; Stich *et al.*, 2013; Massa *et al.*, 2015).

Linkage-based QTL mapping

Linkage-based QTL mapping relies on the principle that DNA sequences that contain the gene of interest and a marker(s) tightly linked to the gene of interest are likely to be inherited together on a chromosome during the meiosis phase of sexual reproduction. In other words, the low recombination frequency ($\ll 50\%$) of two markers indicates that the two markers are tightly linked on a chromosome and are likely to be inherited together (Collard & Mackill, 2008). Linkage-based QTL analysis requires a segregating population (mapping population), molecular markers that cover moderately to high density of the genome and phenotypic information on the mapping population (Collard & Mackill, 2008).

A mapping population can be defined as lines or progeny generated by crossing two or more parents that differ for one or more traits that are under genetic control (Gebhardt *et al.*, 2006). For example, the biparental mapping population can be derived from recombinant inbred lines (RILs), backcross lines (BC), an F_2 population, double haploid lines (DH), near isogenic line (NILs) or an F_1 population derived from heterozygous parents. For potato biparental linkage mapping, a mapping population is developed through selection of two heterozygous parental clones with contrasting effects for the trait of interest, crossing of the two parents and subsequent fixation of the F_1 progeny via clonal maintenance (Gebhardt *et al.*, 2006).

The next step is to phenotype and genotype the mapping population and the parents. The genotypic data generated from the mapping population are used to construct a linkage map, consisting of the position and relative genetic distances between markers on a chromosome (Collard & Mackill, 2008). Once linkage maps are constructed and

the mapping population is phenotyped for the trait(s) of interest, QTL detection is performed by associating the linkage maps with the phenotypic data.

Unlike the situation for diploid species, genetic mapping in autotetraploid species, including tetraploid potato, is not well developed because of the complex segregation patterns (Voorrips *et al.*, 2011). The F₁ progeny carry one to four different alleles per locus. For a bi-allelic locus, a genotype carries one of five possible combinations: homozygous AAAA or BBBB, or heterozygous ABBB, AABB or AAAB. Until recently, genetic mapping in tetraploid potato was limited to a few hundred markers and markers with simplex.(AAAB) by nulliplex (AAAA) configurations (Hackett & Luo, 2003). However, recent advances in SNP calling using the mixture model (Voorrips *et al.*, 2011), marker ordering (Preedy & Hackett, 2016) and the ability to incorporate all dosage information in the map construction using TetraploidSNPMap allow the utilization of heterozygous SNP markers in QTL analysis (Hackett *et al.*, 2013, 2014, 2017; Massa *et al.*, 2015).

Association mapping

Association mapping is an alternative method of QTL detection in a wide range of germplasm collections (Stich *et al.*, 2013; Wu *et al.*, 2015). It is based on linkage disequilibrium (LD) between molecular markers and functional loci in a set of germplasm with unknown ancestry. It shares the same concept with linkage mapping, as both are based on LD. However, association mapping is based on germplasm with historical recombinant events (thousands of generations). Association mapping has some advantages over linkage mapping: it usually results in higher-resolution QTL mapping, it interrogates a broader genetic base and consequently is not restricted to alleles segregating from only two parents, and it involves less time for mapping population development (there is no need of mating design). However, it is limited by a lower power to detect QTLs in a genome scan, substructure of population mapping, and a requirement for high-density markers (Stich *et al.*, 2013; Wu *et al.*, 2015). The decision as to which approach to use, linkage-based QTL mapping or association mapping depends on the goals of the experiment, and in some cases, a combined approach may be called for.

2.2.5.3.Marker-Assisted Selection (MAS)

MAS is a technique that uses genetic variation at the DNA level to track and monitor specific region (s) of the genomes during selection and crossing (Dekkers, 2002; Collard & Mackill, 2008). MAS uses a specific genotype, chromosomal banding, a DNA or RNA motif, or a chemical tag that associates with the desirable traits. A molecular marker closely linked to the locus of the desirable gene can be used to predict whether an individual plant has the desired allele or not. Development of molecular markers for MAS must pass the necessary steps including QTL identification and marker validation in breeding materials (Collard & Mackill, 2008).

MAS is likely to be more reliable, more convenient, or cheaper than phenotype-based selection, and provides the only method for gene pyramiding (Dekkers, 2002; Barone, 2004; Gebhardt *et al.*, 2006; Collard & Mackill, 2008; Tiwari *et al.*, 2013; Ramakrishnan *et al.*, 2015). The combination of reliable phenotyping and MAS has been particularly important in transferring desirable alleles by simple backcrossing into elite germplasm (Dekkers, 2002; Barone, 2004; Gebhardt *et al.*, 2006; Collard & Mackill, 2008; Tiwari *et al.*, 2013; Ramakrishnan *et al.*, 2015).

MAS can increase the efficiency and precision of potato breeding, especially introgression of a resistance gene or simultaneous selection of plants with several traits. It has been applied to major traits like resistance to late blight or cyst nematodes, maturity and others (Barone, 2004; Gebhardt *et al.*, 2006; Tiwari *et al.*, 2013; Ramakrishnan *et al.*, 2015).

The major limitations of MAS are the demands for time, skilled personnel and resources (Barone, 2004; Gebhardt *et al.*, 2006; Tiwari *et al.*, 2013; Ramakrishnan *et al.*, 2015). Furthermore, MAS is applicable only for major effect QTL or genes. However, most agronomic traits exhibit polygenic inheritance, where large numbers of minor effect QTLs are involved in controlling the traits of interest. Hence, MAS has not been as successful as expected by plant breeding communities. To overcome the limitations of MAS, genomic selection has recently been applied to selection for quantitative traits (Stich *et al.*, 2013; Wu *et al.*, 2015). Genomic selection estimates breeding values (GEBVs) of individuals, using a model built with genome-wide markers that have been assayed on a training population representative of the germplasm to be selected subsequently and which has been extensively phenotyped for the target traits. However,

the problem associated with phenotypic selection remains a challenge for genomic selection. In addition to this, genomic selection needs ultra-dense marker distribution while the costs of genotyping remain high for routine breeding activities. In general, potato breeding and genetics, as for other crops, has shown progress in utilization of genomic tools to accelerate breeding activities and it is expected that MAS and genomic-assisted selection, or a combined approach, will help to select for both qualitative and quantitative traits simultaneously in breeding programs in the future.

2.3. Variation in Cd and Zn concentrations between or within plants

Plants differ in Cd and Zn uptake from the soil by the roots and their partition to the different organs including the edible plant portions (Broadley *et al.*, 2007; Grant *et al.*, 2008; White & Broadley, 2009, 2011; Clemens *et al.*, 2013). Factors such as root uptake, root-to-shoot translocation and partitioning between organs affect the final Cd and Zn concentrations in the edible plant portions (Cieřliński *et al.*, 1998; Hart *et al.*, 1998; Harris & Taylor, 2001, 2004, 2013; Genc *et al.*, 2006; Uraguchi *et al.*, 2009; Ueno *et al.*, 2010; Arduini *et al.*, 2014; Kubo *et al.*, 2016; Perrier *et al.*, 2016). All these processes contribute to the differences in uptake and partitioning of Cd and Zn between and within species. It is, therefore, important to identify the most important factors which account for most of the phenotypic variation for a varietal selection program.

2.3.1. Inter-species variation in Cd and Zn accumulations

Variation in Cd (Kuboi *et al.*, 1986; Grant *et al.*, 2008; Yang *et al.*, 2009) and Zn (Broadley *et al.*, 2007; White & Broadley, 2009, 2011) accumulation among species has been reported. Kuboi *et al.* (1986) examined shoot Cd accumulation by 34 plant species grown on a sandy soil with various concentrations of added Cd. According to the authors, the 34 species of plants could be grouped into three general categories: low accumulators (Fabaceae), moderate accumulators (Poaceae, Liliaceae, Cucurbitaceae and Apiaceae) and high accumulators (Solanaceae, Amaranthaceae, Brassicaceae and Asteraceae). However, this classification, which was carried out with regard to facilitating Cd phytoextraction, considered Cd accumulation in the shoot but did not consider the Cd concentration in the edible plant parts. For example, potato belongs to the Solanaceae family, which accumulates large amounts of Cd in the shoot. However, previous studies have shown that the tuber Cd concentration is a very low fraction (one-tenth) of the shoot Cd concentration (Dunbar *et al.*, 2003; Xu *et al.*, 2013; Chen *et al.*, 2014).

Yang *et al.* (2009) measured Cd concentration in the edible portion of six vegetable species (Chinese leek, pakchoi, carrot, radish, tomato and cucumber) and found that Cd concentration in the edible parts ranged from 0.05 to 0.2 mg kg⁻¹ (0.05 mg kg⁻¹ for cucumber and tomato, 0.1 mg kg⁻¹ for carrot and radish, and 0.2 mg kg⁻¹ for pakchoi and leek). Of course, Cd concentration comparisons between species may be either over- or under-estimated since there is considerable variation within the same species (Kuboi *et al.*, 1986; Yang *et al.*, 2009). Therefore, cultivar selection for this kind of study can determine the outcome of the comparison.

From a practical point of view, comparisons of Cd accumulation in related species can have significant implications for crop improvement by identifying sources of genetic material or grafting material, particularly for horticultural crops (Arao *et al.*, 2008). According to Arao *et al.* (2008), aubergine (*Solanum melongena*) has greater Cd concentration in the fruits compared to the related species, *Solanum torvum*. Grafting the scion of *Solanum melongena* onto the rootstock of *Solanum torvum* reduced eggplant fruit Cd concentrations by 63-74% in plants grown in Cd-polluted and unpolluted soil, compared with grafting onto *Solanum melongena* rootstock. It was also observed that the shoot Cd concentration in *S. torvum* was less than that in *S. melongena*. The authors suggested that variation in root-to-shoot translocation of Cd occurred between the two *Solanum* species.

With respect to Zn inter-species variation, a comprehensive meta-analysis of 1108 studies comparing shoot Zn concentration among 365 plant species from 48 plant families and 12 key angiosperm clades showed that the least shoot Zn concentrations were exhibited in the Linaceae, Poaceae and Solanaceae while the largest shoot Zn concentrations were recorded in the Brassicaceae, Amaranthaceae, and Salicaceae (Broadley *et al.*, 2007). In general, leafy vegetables, followed by legumes, exhibited greater Zn concentration than did grains or tuber crops (White and Broadley 2011 and references therein).

2.3.2. Intra-species variation in Cd and Zn concentrations in edible organs

Breeding for decreased Cd and increased Zn concentrations in any crop, including potato, as for any heritable trait, requires the availability of genetic variation for the trait to exist in breeding germplasm (Jegadeesan *et al.*, 2010; Wu *et al.*, 2015). Studies showing genetic variation for Cd concentrations in edible portions of different crops

have included wheat (Cieřliński *et al.*, 1998; Harris & Taylor, 2001, 2004, 2013; Stolt *et al.*, 2003; Hart *et al.*, 2006; Knox *et al.*, 2009; Wiebe *et al.*, 2010); rice (Liu *et al.*, 2007; Ueno *et al.*, 2010; Uraguchi *et al.*, 2014); sweet potato (Huang *et al.*, 2015; Xin *et al.*, 2017); soybean (Benitez *et al.*, 2010; Jegadeesan *et al.*, 2010); barley (Wu *et al.*, 2015) and radish (Xu *et al.*, 2012b).

With respect to Zn concentration, genetic variation in edible portions has been reported for cereals (Monasterio & Graham, 2000; Menkir, 2008; Chatzav *et al.*, 2010; Zhang *et al.*, 2011; Sadeghzadeh *et al.*, 2015; Velu *et al.*, 2017); legumes (Blair *et al.*, 2009; Baloch *et al.*, 2014); leafy vegetables (Wu *et al.*, 2007) and root crops (Nicolle *et al.*, 2004; Chávez *et al.*, 2005).

Monasterio and Graham (2000) evaluated 132 wheat accessions and found grain Zn concentrations ranging from 25.2 to 53.3 mg kg⁻¹. Similarly, Chatzav *et al.* (2010) assessed 154 wild emmer accessions and reported Zn concentration varying from 45 to 90 mg kg⁻¹. Substantial variation in grain Zn concentration was also reported in maize inbred lines with values ranging from 14 to 45 mg kg⁻¹ (Menkir, 2008).

In root crops, Chávez *et al.* (2005) evaluated 600 cassava genotypes and found that root Zn concentration varied from 2.63 to 37.52 mg kg⁻¹, which is a 14-fold variation among the genotypes. Likewise, Nicolle *et al.* (2004) evaluated 20 carrot genotypes and reported Zn concentration in the roots ranging from 17 to 29 mg kg⁻¹, almost a 2-fold variation among genotypes.

In legume crops, Blair *et al.* (2009) evaluated 87 common bean genotypes and reported seed Zn concentrations ranging from 17 to 37 mg kg⁻¹. In another study, Baloch *et al.* (2014) evaluated 129 Turkish faba bean landraces and found that Zn concentration in the seeds ranged from 10.4 to 49.3 mg kg⁻¹.

2.3.3. Physiological basis of Cd and Zn concentrations in edible portions

As reviewed in section 2.3.2, genotypic variations for Cd and Zn concentrations have been reported in many crops. However, identification of the physiological basis of such differences in Cd or Zn concentration between genotypes in the literature is limited. In this review, I have focused mainly on research findings in wheat and rice, where the mechanisms underlying such variation have been examined in detail.

Hart *et al.* (1998) and Harris and Taylor (2001, 2004, 2013) found that differences in grain Cd concentration between durum wheat cultivars were associated with variations in root-to-shoot translocation. The authors suggested that the genotypes may differ with respect to root Cd vacuolar sequestration. In line with root Cd vacuolar sequestration, Stolt *et al.* (2003) conducted an experiment to determine whether wheat cultivars differed with respect to root internal phytochelatin chain-length distribution and phytochelatin thiol: Cd molar ratio between wheat cultivars. These authors did not find significant differences in types and distributions of phytochelatins between the cultivars. Hart *et al.* (2006) also reported that phytochelatin was not the limiting factor for root Cd sequestration in wheat. The authors suggested that differential grain Cd concentration in wheat was not associated with phytochelatin-based root traits but with variable capacity of the cultivars to transport Cd into the vacuole.

Cieśliński *et al.* (1998) evaluated durum wheat cultivars for LMWOA production and found that cultivars that accumulated large Cd concentration produced larger amount of LMWOAs than did cultivars that accumulated smaller Cd concentrations. The authors suggested that LMWOAs increased phyto-available Cd in the rhizosphere and thereby enhanced plant Cd uptake. Recent studies by Arduini *et al.* (2014), Perrier *et al.* (2016) and Kubo *et al.* (2016) reported significant differences among wheat cultivars in terms of root Cd uptake. These studies also reported that shoot biomass can influence shoot and grain Cd concentration through dilution.

It is generally accepted that the physiological basis of differences in grain Cd concentration in wheat depends on the test materials being used and involve multiple factors including root-to-shoot translocation (Hart *et al.*, 1998; Harris & Taylor, 2001, 2004, 2013), Cd uptake (Arduini *et al.*, 2014; Kubo *et al.*, 2016; Perrier *et al.*, 2016) and shoot-grain partitioning (Arduini *et al.*, 2014; Kubo *et al.*, 2016; Perrier *et al.*, 2016).

In rice, Liu *et al.* (2007) compared two rice cultivars differing with respect to Cd uptake, and found that the two cultivars differed in root exudation of LMWOAs. According to this study, the cultivar that accumulated greater Cd concentrations produced an increased amount of LMWOA than did the cultivar that accumulated lower Cd concentrations. Among the LMWOAs, acetic acid and formic acid constituted more than 96% of the total concentration, while citric acid constituted only about 0.1%. The authors suggested that LMWOA secretion by rice roots may be one

of the mechanisms determining differences in plant Cd uptake properties of rice cultivars.

A study by Uraguchi *et al.* (2009) found that root-to-shoot Cd translocation is a limiting factor for differences in shoot and grain Cd accumulation in rice. Ueno *et al.* (2010) identified a transporter gene in rice, *OsHMA3*, responsible for transport of Cd to the root vacuoles, associated with differences between rice cultivars for root Cd sequestration. Loss of the function of an *OsHMA3* gene resulted in greater shoot and grain Cd accumulation. Another study by Uraguchi *et al.* (2014) also reported that shoot-to-grain Cd remobilization in rice was highly influenced by the *OsICT1* gene and overexpression of the *OsLCT1* gene resulted in an increased Cd concentration in the grain. Based on these results, it is reasonable to conclude that more than two factors are involved in the determination of grain Cd concentration in rice.

With reference to Zn concentration in edible portions, considerable numbers of studies are available in wheat, rice and other crops. The studies suggest that inter-cultivar differences in root uptake (Genc *et al.*, 2006; Jiang *et al.*, 2007; Impa *et al.*, 2013), root-to-shoot translocation (Hart *et al.*, 1998; Wu *et al.*, 2010; Impa *et al.*, 2013) and partitioning between shoot and edible portions including grains and fruits (Genc *et al.*, 2006; Kato *et al.*, 2010; Wu *et al.*, 2010; Yamaji & Ma, 2014) are possible factors determining difference in Zn concentration in the edible plant portions.

2.3.4. QTL mapping for Cd and Zn concentrations in edible portions

The genetic basis of differences in the accumulation and/or uptake of Cd and Zn among cultivars has been investigated using QTL mapping (Knox *et al.*, 2009; Wiebe *et al.*, 2010; (Wu *et al.*, 2015). As outlined in section 2.2.5, advances in plant genomics allow the use of high-throughput genotyping platforms to construct ultra-dense genetic maps to localise genes/QTLs that control the accumulation of or tolerance to elements, including Cd and Zn.

To date, QTL analysis of Cd concentration in edible plant parts has been conducted for many crops, including grain of wheat (Knox *et al.*, 2009; Wiebe *et al.*, 2010), seed of soybean (Benitez *et al.*, 2010; Jegadeesan *et al.*, 2010), grain of barley (Wu *et al.*, 2015) and radish root and shoot (Xu *et al.*, 2012b).

In soybean, biparental linkage QTL mapping, using crosses between Canadian cultivars which differed with respect to Cd accumulation, identified a major QTL on chromosome 9 (Jegadeesan *et al.*, 2010). This QTL accounted for 57% of the phenotypic variation in seed Cd concentration and was named the *Cda1* locus (Jegadeesan *et al.*, 2010). An independent study (Benitez *et al.*, 2010) also confirmed the identification of the same QTL on chromosome 9 using a recombinant inbred line (RIL) population derived from a cross between one Canadian and one Japanese cultivar and named it as *cd1*. Combining the QTL position with the soybean genome sequence suggested that *cd1* was located in the vicinity of a *PIB-ATPase* gene, which is an important gene for encoding proteins which transport cations across the cell membrane. Molecular markers linked to the *cd1* QTL have played a role in the identification of cultivars which accumulate increased concentrations of Cd (Benitez *et al.*, 2010; Jegadeesan *et al.*, 2010).

In barley, a genome-wide association study by Wu *et al.* (2015) found QTLs which explained between 3-13% of the phenotypic variation observed in the population, indicating the complex inheritance of grain Cd concentration. Furthermore, the study observed positive and significant associations between the accumulation of Cd and Zn in grain.

In durum wheat, an earlier study by Penner *et al.* (1995) identified a dominant RAPD marker which was linked in coupling to the high-Cd *Cdu1* allele. This marker has been applied to a MAS programme. However, the RAPD marker was linked in repulsion to the low-Cd allele of this gene, which limited its utility in a marker-assisted backcross programme. For the sake of developing co-dominant markers and to pave the way towards map-based cloning, Knox *et al.* (2009) conducted QTL mapping, using simple sequence repeat (SSR) markers and developed markers for selecting individual accumulating decreased Cd concentrations.

In wheat and soybean, the trait of Cd accumulation is under the control of a single locus, apparently with a single dominant allele for the tendency not to accumulate Cd in seeds/grains (Clarke *et al.*, 1997; Knox *et al.*, 2009; Jegadeesan *et al.*, 2010). In rice, the situation is slightly more complex, but the trait seems to be under the control of a relatively large-effect QTL (Xue *et al.*, 2009; Zhang *et al.*, 2011; Yan *et al.*, 2013). In the case of barley, the association mapping by Wu *et al.* (2015) showed that the inheritance of grain Cd concentration was quantitative in nature.

Similarly, the genetic control of Zn accumulation in edible plant portions has been mapped in different crops, including wheat (Genc *et al.*, 2009; AbuHammad *et al.*, 2016; Velu *et al.*, 2017), barley (Sadeghzadeh *et al.*, 2015; Hussain *et al.*, 2016; Reuscher *et al.*, 2016), common bean (Blair *et al.*, 2009), maize (Zhang *et al.*, 2017) and rice (Zhang *et al.*, 2011). The trends of inheritance vary between crop species, from oligogenic in common beans and barley (Blair *et al.*, 2009; Sadeghzadeh *et al.*, 2015) to polygenic in rice and wheat (Zhang *et al.*, 2011).

2.4. Physiological and genetic mechanisms underlying differences in tuber Cd and Zn concentrations in potato

An earlier study (Harris *et al.*, 1981) reported the absence of significant differences in tuber Cd concentration among cultivars grown on Cd-contaminated soil. However, later studies (McLaughlin *et al.*, 1994, 1997; Dunbar *et al.*, 2003) showed significant differences among potato cultivars with respect to tuber Cd concentration. Despite genotypic variation among potato cultivars with respect to tuber Cd concentrations, limited information is available on the physiological basis of differences in tuber Cd concentration (Dunbar *et al.*, 2003). Dunbar *et al.* (2003) compared two Australian potato cultivars ('Kennebec' and 'Wilwash'), for tuber Cd concentration differences under hydroponic conditions and found that the two cultivars differed in Cd partitioning between root and tuber. However, the two cultivars accumulated the same content of plant Cd. To date, no genetic information is publicly available about potato tuber Cd concentration.

Genotypic variability in tuber Zn concentration has also been reported (Andre *et al.*, 2007; Burgos *et al.*, 2007; White *et al.*, 2009, 2017; Brown *et al.*, 2011; Abebe *et al.*, 2012; Subramanian, 2012; Kromann *et al.*, 2017). Andre *et al.* (2007) evaluated 74 landraces and found that tuber Zn concentration ranged from 12.6 to 28.83 mg kg⁻¹. Similarly, Burgos *et al.* (2007) assessed 37 accessions and found that tuber Zn concentration varied from 8 to 20 mg kg⁻¹. Furthermore, Abebe *et al.* (2012) and Brown *et al.* (2011) reported genetic variation among potato cultivars, varying from 7 to 20 mg kg⁻¹. Similar levels of genotypic variation have also been reported in Scottish (White *et al.*, 2009, 2017; Subramanian, 2012) and Andean cultivars (Kromann *et al.*, 2017). To date, only one publication has reported information on the genetic control of tuber Zn concentration (Subramanian, 2012). However, no

information is available on the physiological mechanism of determining differences in Zn concentration in the tubers.

Despite the importance of potato in terms of both food security and nutritional values, limited information is available on the genetic and physiological mechanisms underlying tuber Cd and Zn concentrations (Dunbar *et al.*, 2003; Bradshaw *et al.*, 2006). Furthermore, there is no information published on any correlation between Cd and Zn concentrations in tubers of potato.

Preliminary evaluation of 14 commercial potato cultivars commonly grown in Ireland showed significant differences in Cd and Zn tuber concentrations under both field and pot conditions (Teagasc, unpublished data). However, the physiological mechanisms underlying the differences in tuber Cd and Zn concentrations among the cultivars are unknown. Among the cultivars tested, ‘Cara’ and ‘Lady Rosetta’ exhibited the least and the largest concentrations of both Cd and Zn in the tubers, respectively. The two cultivars also differed widely with respect to maturity class and root system size. ‘Cara’ is characterized by late maturity, an indeterminate growth habit, long roots and large root biomass, while ‘Lady Rosetta’ is early maturing, a determinate growth habit and short seminal root (The European Cultivated Potato Database, 2017). These two cultivars were selected for use in this research to study the physiological and genetic mechanisms of differences in tuber Cd and Zn concentration. Previous studies have also shown that Lady Rosetta has a larger Cd accumulation capacity in tubers (Olsson and Roslund, 1999) and that Cara tends to have less trace transition metal concentrations in tubers than other cultivars (Rivero *et al.*, 2003; Luis *et al.*, 2011).

By considering these previous studies, three separate but connected subjects were examined in this thesis. The first experimental chapter (Chapter 3) addressed the main physiological mechanisms underlying differences in tuber Cd and Zn concentrations and the sources of phloem Cd and Zn supply to the tubers, using the two cultivars. The second research chapter (Chapter 3) was conducted with the aim of identifying the most important physiological factors underlying differences in tuber Cd and Zn concentrations, by studying reciprocal grafting between the two cultivars and the phenotypes of selected F₁ genotypes from the mapping population. The aim of the third experimental chapter (Chapter 5) was to investigate the genetic basis of the observed differences in tuber Cd and Zn concentrations, using a F₁ mapping population derived from a cross between the two cultivars used in the first two experiments.

2.5. Overall questions and objectives of the research described in this thesis

1. Does Cd and Zn accumulation vary over time and among organs between the two cultivars?

To investigate the temporal pattern of accumulation of Cd and Zn between Cara and Lady Rosetta, the two cultivars were assessed for dry weight accumulation, Cd and Zn concentration and content in different organs over the growth period.

2. Is tuber Cd and Zn concentration regulated by few or many genes?

To identify QTLs controlling Cd and Zn concentration in the tubers, a mapping population consisting of 188 F₁ plants from a cross between Cara and Lady Rosetta, were developed, genotyped using the SolCap 8303 SNP markers and phenotyped over two years. The QTLs responsible for differences in tuber Cd and Zn concentrations were identified.

3. Do roots or shoots control Cd and Zn accumulation in the tuber of potato?

To answer this question, reciprocally grafted plants, involving grafts between Cara and Lady Rosetta, and selected F₁ genotypes were examined for dry weight, and Cd and Zn concentrations and contents of different organs.

Chapter 3: Cadmium and zinc uptake and partitioning in potato

Mengist, M.F., Milbourne, D., Griffin, D., McLaughlin, M.J., Creedon, J., Jones, P.W. and Alves, S., 2017. Cadmium uptake and partitioning in potato (*Solanum tuberosum* L.) cultivars with different tuber-Cd concentration. *Environmental Science and Pollution Research*, 24: 27384–27391.

Mengist, M.F., Milbourne, D., Griffin, D., McLaughlin, M.J., Jones, P.W. and Alves, S., 2018. Zinc uptake and partitioning in potato cultivars during growth and development. Final stage for submission.

3.1. Introduction

Unlike cereal or legume crops, potato has special features in which the tubers are connected to stolons and stolon roots. Therefore, it is important to know the role of tubers, stolons and stolon roots in tuber Cd and Zn accumulation. Earlier studies (Kratzke & Palta, 1985, 1986) showed that tubers do not have direct xylem connection with basal roots. A study by Reid *et al.* (2003) proved that the tubers and their associated stolon and stolon roots contribute only a minor fraction of the overall Cd absorbed by the plant. According to the authors, the majority of Cd is taken up by the basal roots, transported to the shoot *via* the xylem and then redistributed to the tubers *via* the phloem pathway. Furthermore, studies showed variation between cultivars for tuber Cd (McLaughlin *et al.*, 1994, 1997; Dunbar *et al.*, 2003; Larsson Jönsson & Asp, 2011) and Zn concentration (Tekalign & Hammes, 2005; Andre *et al.*, 2007; Burgos *et al.*, 2007; Brown *et al.*, 2011; Abebe *et al.*, 2012; Subramanian, 2012; White *et al.*, 2012, 2017). However, limited information is available on the physiological mechanisms underlying differences in tuber Cd concentration (Dunbar *et al.*, 2003), while there is no literature available regarding the physiological basis of differences in tuber Zn concentration.

For this specific study, as noted in section 2.4, the two cultivars, Cara and Lady Rosetta, were chosen from 14 commercial cultivars commonly grown in Ireland. Hence, the objectives of this study were to determine the possible physiological bases of differences in tuber Cd and Zn concentrations. To study these phenomenon, Cara and Lady Rosetta were assessed for dry weight, and for Cd and Zn concentrations between organs across the plant life cycle.

3.2. Materials and Methods

3.2.1. Plant materials and growth conditions

The experiment was conducted outdoors in 15 L pots, each filled with 18 kg of soil at Oak Park, Carlow, Ireland. The soil used was sourced in 2014 from a field (3.5 ha, Grange, Ireland) known to have naturally high levels of Cd (of geogenic origin) from a previous sampling campaign, covering most of the fields on this farm. Soil samples were acquired with an agricultural soil auger for determination of the soil nutrient amounts for fertiliser advice, consisting of a sub-sample of a representative soil sample from the entire field taken in a grid pattern and comprising a minimum of 20 soil cores

at c. 10 cm depth. About 0.25 to 0.5 kg soil were obtained for analysis and thoroughly mixed before sub-sampling. Selected soil characteristics are presented in Table 3.1. The soil was air dried, crushed, mixed well and placed in 15 L pots. The soil was supplemented by 25 g NPK(S) (7-6-17(13)) (Goulding Fertilisers, Cork, Ireland) per pot basis. Equal-sized tubers of both cultivars were selected and sprouted for three weeks before planting (one tuber per pot) in May 2014. The experiment was laid out in a randomized complete block design (RCBD) with two cultivars, three replications and nine sampling points. Water was supplied by drip irrigation. This experiment was repeated in 2015 with similar conditions that of the first-year experiment except for second-year experiment only five sampling points, 30 days to days to senescence, were considered.

3.2.2. Plant harvesting and sampling

A total of nine sampling time points were selected from early in the growth period to senescence. The first sampling was at 30 days after planting (DAP) and sampling continued approximately every two weeks thereafter. At each harvest, plants were carefully separated from the soil. The tubers were collected, washed with water, soaked for 10 minutes in an ice-cold 5 mM solution of CaCl_2 and rinsed in distilled water to remove exogenous Cd or Zn. Roots of the intact plant were washed carefully with water, immersed for 10 min in an ice-cold 5 mM CaCl_2 solution and rinsed in distilled water while still attached to their shoots (Dunbar *et al.*, 2003). Then, the plants were detopped at the base of the stem and further divided into young leaves (the first four leaves from the growing apex) and old leaves (the remaining leaves) and stem. Young leaves were sampled until 73 and 98 DAP for Lady Rosetta and Cara, respectively, based on initiation of senescence. Fresh weights of all the tissues were determined immediately after collection and samples were oven dried at 70°C until a constant weight was attained. The samples were placed in 50 ml centrifuge tubes with stainless steel balls and ground using a ball mill (Retsch™ MM 400 Mixer Mill, Retsch GmbH, Hann, Germany). Between samples, the stainless-steel balls were carefully washed with distilled water and dried. Afterwards, samples were stored until further analysis.

3.2.3. Cd and Zn analysis

Dried organ powder of approximately 0.2 g tuber or 0.1 g leaf, stem or root was microwave digested (MARS 5, CEM Corporation, Matthews, NC, USA) with 1 ml ultrapure water (resistivity 18 $\text{M}\Omega\text{cm}^{-1}$), 1 ml concentrated nitric acid (HNO_3 , 69%,

Aristar grade, VWR, Radnor, USA) and 0.5 ml hydrogen peroxide (H₂O₂. 30%, Sigma-Aldrich, Missouri, USA). The digested sample was diluted with ultrapure water to 50 ml in a plastic volumetric flask, transferred to 50 ml centrifuge tube and kept at 4°C prior to chemical analysis. The samples were analysed by inductively coupled plasma mass spectrometry (ICP-MS) (Perkin Elmer ELAN DRC-e, Monza, Italy). Every digestion batch (40 samples) included two reagent blanks, Certified Reference Material (CRM) or Control Material (CM) and one sample duplicate. Reagent blanks verified the absence of contamination and were always below 0.05 µg/ml. The CRM used was strawberry leaves (LGC7162) and the CM was carrot root powder (IC-CS-CR-2), with recoveries between 85 and 105%. Relative difference between duplicates was always less than 20%. The microwave digestion programme is presented in Table 3.2.

3.2.4. Statistical analysis

Two-way analysis of variance (ANOVA) was applied to evaluate differences in dry weight, Cd and Zn concentration, and Cd and Zn content between the two cultivars. Analysis of simple effects for significant interaction between time and cultivar was performed by SAS slice procedure (SAS 9.4). Multiple comparison differences between cultivars were determined by the Tukey studentized t-test.

Table 3.1 Selected chemical and physical characteristics of the soil

Cd total ^a	1.0 mg kg ⁻¹
Zn total ^a	73 mg kg ⁻¹
Extractable Cd ^b	0.9 mg L ⁻¹
Extractable Zn ^b	4.3 mg L ⁻¹
OM	7.4 %
pH	5.4
K ^c	74 mg L ⁻¹
P ^c	2.9 mg L ⁻¹
Sand	34%
Silt	55%
Clay	11%

^aExtracted with aqua-regia; ^bExtracted with 0.5 M EDTA solution, at pH 7;

^cExtracted by Morgan's solution. Analyses were conducted in a soil testing laboratory accredited by the Irish National Accreditation Board.

Table 3.2 Microwave programme for digestion of potato plant organs

Stage	Power (Watts)		Ramp (mins)	Temperature (°C)	Hold time (mins)
	Max	%			
1	1600	25	01:00	100	01:00
2	1600	50	02:00	180	02:00
3	1600	70	00:00	180	10:00
4	400	100	10:00	100	00:00
5	400	0	00:00	0	10:00

Developed in The State Laboratory, Backweston Laboratory Campus, Celbridge, Co. Kildare, W23 VW2C, Ireland.

3.2.5. Data handling: partition quotient and weight-normalized whole plant Cd and Zn concentration calculations

Partition quotient (PQ) and weight-normalized whole plant Cd and Zn concentrations were calculated as described by Waters and Grusak (2008). For PQ, dry weight (DW) of each organ was calculated as a percentage of total plant weight at each time point and Cd content of each organ was also calculated as percentage of total plant Cd content at each time point. Similarly, Zn content of each organ was also calculated as percentage of total plant Zn content at each time point. Using these values, the normalized partitioning of Cd within the plant was calculated by dividing each organ's percentage Cd content by its percentage DW, and multiplying by 100, which is referred to as the PQ. For Zn, the normalized partitioning of Zn within the plant was calculated by dividing each organ's percentage Zn content by its percentage DW and multiplying by 100. Normalized whole plant Cd concentration was calculated by dividing whole plant Cd content at each time point by its whole plant DW at that time point for both cultivars. Similarly, normalized whole plant Zn concentration was calculated by dividing whole plant Zn content at each time point by its whole plant DW at that time point for both cultivars. For estimation of Cd content loss from non-tuber organs, the final Cd content was subtracted from the prior time point which had the largest Cd content; net loss was compared with the final tuber content to determine the contribution of remobilized minerals to tuber Cd content. Similar formula also applied to estimate Zn remobilization from non-tuber organs (Waters and Grusak 2008).

3.3. Results

3.3.1. Plant growth and biomass production

The first harvest was carried out at 30 days after planting. At this stage, the root system had developed well. Lady Rosetta had begun developing tubers while Cara was in the tuber initiation stage. At this stage, no significant differences between the two cultivars were observed with respect to dry weight or growth. In the subsequent harvests, total dry weight *per plant* was significantly influenced by cultivar ($P<0.05$), with Cara producing heavier plants than Lady Rosetta, and time ($P<0.01$). However, the results did not show significant interaction between cultivar and time of harvest (Appendix-Table 8.1). There was an overall increase in biomass accumulation for each cultivar until 98 DAP (Figure 3.1). Afterwards, the biomass remained constant with no statistical differences for each cultivar (Appendix-Table 8.2). The contributions of each organ to the total biomass over time are shown in Figure 3.2.

Overall root dry weight accumulation was significantly ($P<0.01$) affected by time, cultivar and interaction between the two factors (Appendix-Table 8.1, Table 3.3). The pattern of root biomass accumulation was similar between the two cultivars, increasing until 45 or 57 DAP for Lady Rosetta or Cara, respectively, and exhibiting a slow decline afterwards. From 45 DAP until final harvest, Cara had significantly ($P<0.01$) more root biomass, attaining a maximum difference than Lady Rosetta at 57 DAP (*c.* two-fold more) as presented in Figure 3.2B.

The data showed that stem and leaf biomass accumulation was greatly affected by cultivar, time and their interaction (Appendix-Table 8.1). Stem and leaf biomass accumulation followed a similar pattern in Lady Rosetta, increasing until 57 DAP and slowly declining afterwards (Figures 3.2C, 3.2D). In Cara, stems and leaves exhibited different trends. Leaf biomass increased until 84 DAP and then slowly declined (Figure 3.2D). On the other hand, stem biomass of Cara increased until 57 DAP, remained constant until 110 DAP and decreased afterwards (Figure 3.2C). Cara had consistently and significantly ($P<0.05$) larger stem biomass than Lady Rosetta throughout plant growth, except at 30 DAP. Similarly, Cara accumulated significantly greater leaf dry weight than Lady Rosetta at 84 DAP and thereafter (Figures 3.2C, 3.2D). Details of [time x cultivar] interaction are presented in Appendix-Table 8.3.

Tuber biomass was significantly affected by time ($P<0.05$), cultivar and interaction between the two factors (Appendix-Table 8.1). Tuber biomass increased in both cultivars throughout plant development, although the biomass increment was faster in the early stages of tuber development. Lady Rosetta had accumulated significantly ($P<0.05$) larger tuber dry weight at 73 DAP, while Cara had significantly ($P<0.01$) greater tuber dry weight at 125 DAP and final harvest (Figure 3.2A, Appendix-Table 8.3).

In general, tuber bulking was active between 40 to 100 days of planting for both cultivars (Figure 3.1). At 100 days after planting, Cara had accumulated greater tuber dry weight than Lady Rosetta. When comparing the dry weight of non-tuber organs at final harvest, Cara accumulated 72% more dry weight than Lady Rosetta. Regardless of cultivars, more than 90% of the total dry weight was shifted to the tubers. Overall, Cara had greater total dry weight than Lady Rosetta (Figure 3.1, Table 3.3).

3.3.2. Cd uptake and distribution between organs of the two cultivars

3.3.2.1. Cd concentration in the organs of the two cultivars

The overall data showed that tuber, stem and leaf Cd concentrations were significantly ($P<0.01$) influenced by cultivar, time and the interaction of those factors (Appendix-Table 8.1). However, root Cd concentration was significantly affected by both cultivar and time ($P<0.01$) but no interaction was observed between the two factors (Appendix-Table 8.1).

The pattern of tuber-Cd concentration at the different growth stages for both cultivars is shown in Figure 3.3A. The two cultivars showed statistically significant ($P<0.01$) differences in Cd concentration in tubers at all stages of development. Lady Rosetta consistently exhibited nearly two-fold greater Cd concentration than Cara. The results also showed that Cd concentration in the tubers of Cara did not vary statistically over the growth period. On the other hand, Cd concentration in the tubers of Lady Rosetta revealed significant ($P<0.01$) variation over time (Figure 3.3A, Appendix-Table 8.4), increasing between 57 and 84 DAP and remaining constant thereafter.

The pattern of Cd concentration in stems and leaves also differed between the cultivars (Figure 3.3). For Lady Rosetta, Cd concentration in stems was relatively constant until 73 DAP, increased until 84 DAP and remained constant thereafter (Figure 3.3C). Cd

concentrations in leaves of Lady Rosetta increased until 110 DAP (Figure 3.3D), and remained constant thereafter. For Cara, Cd concentration in stems and leaves was somewhat constant throughout plant growth (Figure 3.3C, 3.3D). Comparing the two cultivars, Cd concentration in stems was greater in Lady Rosetta than in Cara throughout the plant growth period, with a larger difference between cultivars observed after 73 DAP. Cd concentration in the leaves was similar until 84 DAP before becoming statistically different afterwards, with Lady Rosetta exhibiting a greater concentration relative to Cara (Figures 3.3C, 3.3D, Appendix-Table 8.5). New leaves of both cultivars had similar Cd concentrations, which were less than the corresponding values in old leaves until 57 DAP (Figure 3.3D). Cd concentration in the roots sharply decreased over time, with the two cultivars showing a similar pattern until 73 DAP and remained constant afterwards, as presented in Figure 3.3B. Throughout the experiment, Cd concentration in Cara roots was significantly ($P<0.01$) greater than in Lady Rosetta except from 80 to 120 DAP.

It is interesting to note that Cd concentrations in different organs varied greatly between each other and over time (Figure 3.3). At 30 DAP, the Cd-concentration varied in order of roots > stems ~ leaves for Lady Rosetta and Cara, while at final harvest, it varied in order of leaves ~ stems > roots >> tubers for Lady Rosetta and in the order of leaves ~ stems ~ roots >> tubers for Cara (Figure 3.3, Table 3.3).

3.3.2.2.Total Cd uptake and organ distribution of Cd

At final harvest, the two cultivars showed significant ($P<0.05$) differences in total plant Cd accumulation, with an overall plant Cd content about 20% more in Lady Rosetta (Table 3.3). The two cultivars showed a similar pattern for total plant Cd over the growth period, as depicted in Figure 3.4. Accordingly, total Cd content increased in the first 84 DAP and remained constant afterwards. The total Cd content was similar in both cultivars up to 73 DAP except at 57 DAP where Cara had significantly greater Cd concentration than did Lady Rosetta. After 73 DAP, Lady Rosetta exhibited consistently and statistically greater total Cd concentration than Cara (Figure 3.4).

Total plant Cd *per g* root (DW) was much greater in Lady Rosetta than in Cara and the gap increased over time (Figure 3.5), suggesting that a more root-specific mechanism operated as part of the differences in overall Cd uptake between the two cultivars, such

as the release of LMWOAs by root tips into the rhizosphere and/or differences associated with root architecture.

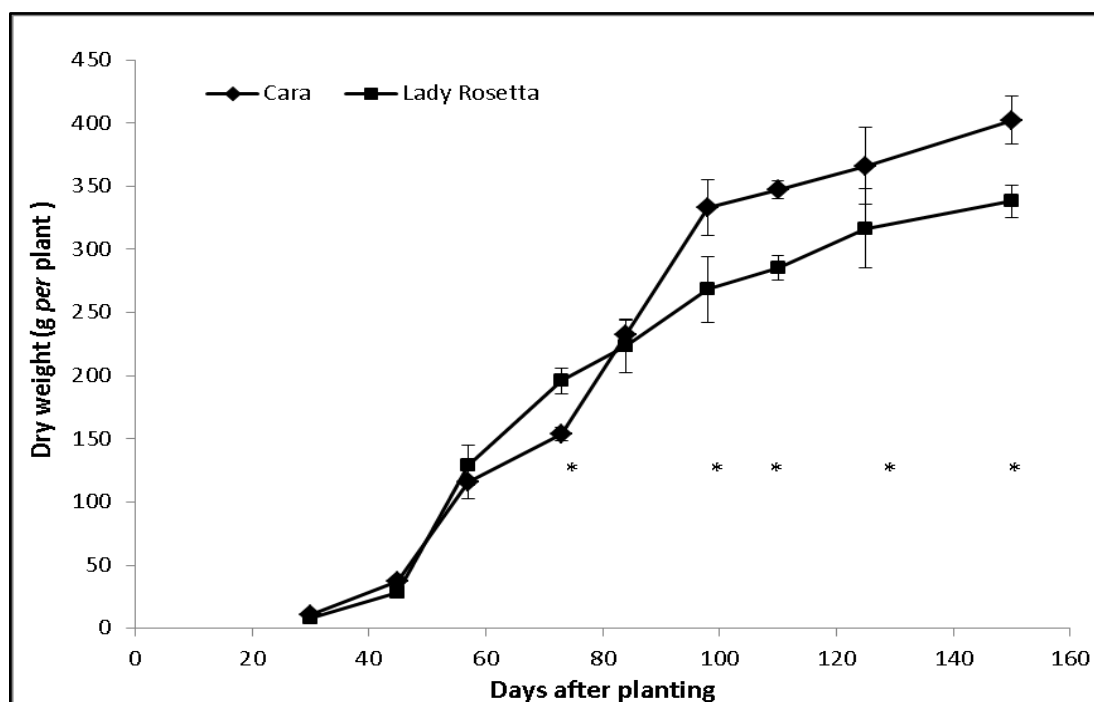


Figure 3.1 Overall biomass accumulation in potato plants over the growth period. Data points represent the mean \pm standard error of the mean (n=3). * Significant ($P < 0.05$) difference between the two cultivars at given harvest time.

Trends of Cd partitioning among organs of the two cultivars are presented in Figure 3.5. At 30 DAP, the two cultivars differed in the partitioning of total Cd content, mainly between the stem and the leaves. The total Cd in Cara was partitioned 24% to the root, 60% to the leaves and 16% to the stem. On the other hand, the total Cd of Lady Rosetta was partitioned 22% to the root, 53% to the leaves and 25% to the stem, with a greater proportion to the stem. Tubers had developed in only Lady Rosetta plants at this stage, but were very small and their contribution to Cd content was negligible (Figure 3.2A).

In Lady Rosetta, between 30 and 45 DAP, Cd partitioning changed dramatically, with a 13% decrease to the roots and 12% increase to the leaves in terms of total Cd content (Figure 3.5). Between 45 and 57 DAP, the main changes in partitioning observed were a sharp increase (18%) in tuber Cd content and a decrease (17%) in Cd content in the leaves. These trends continued at a slower pace between 57 DAP and final harvest. From 84 DAP until final harvest, a trend towards a slow decrease in total Cd content in stems could be perceived. After 73 DAP, the root contribution to total Cd content was $\leq 2\%$ (Figure 3.5).

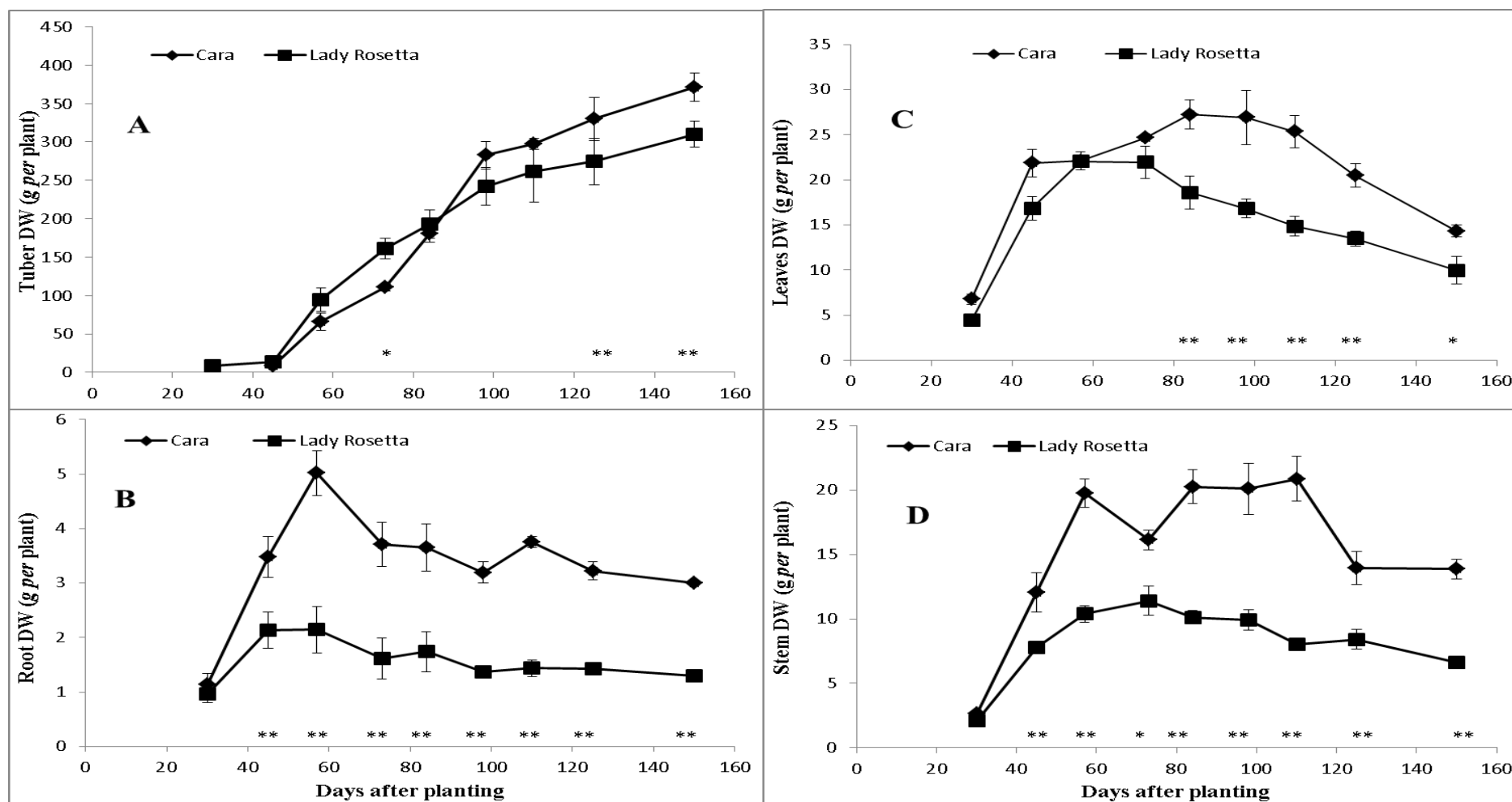


Figure 3.2 DW accumulation of different organs of Cara and Lady Rosetta plants over time. Data points represent the mean \pm standard error of the mean (n=3). **, * Significant at $P < 0.01$ and $P < 0.05$, respectively for two cultivars at a given harvest time.

In Cara, between 30 and 73 DAP, Cd concentration increased in tubers and stems, and decreased in leaves and roots (Table 3.3). Afterwards, the change in tuber Cd concentration was smaller, with root, leaf and stem Cd content decreasing slowly until final harvest (Figure 3.5). At final harvest, the Cd partitioning (%) in Lady Rosetta was 50% in the tubers, 31% in the stems, 18% in the leaves and ~1% in roots, whereas, in Cara, tuber-Cd accounted for only 39% while stems, leaves and root accounted for 36%, 20% and 5%, respectively (Table 3.3).

Despite variation in Cd concentration and DW, leaf and stem Cd contents were comparable between the two cultivars (Figure 3.6). Around 98 DAP, the shoot biomass of Cara was approximately twice that of Lady Rosetta, while the shoot-Cd concentration was about one-half that of Lady Rosetta. These two opposing effects yielded similar Cd content values in the shoots of the two cultivars (Figure 3.6), indicating the importance of shoot biomass in governing the differences in shoot-Cd concentration between the two cultivars.

3.3.2.3. Remobilization of Cd

Remobilization (net loss of Cd) of Cd from vegetative organs (stem and leaf) is shown in Figure 3.7. Net loss of leaf Cd, calculated as final Cd content, expressed as a percentage of the maximum Cd content in that organ, was approximately 32% and 31% for Cara and Lady Rosetta, respectively. Similarly, net loss from stem was approximately 31 and 53% for Cara and Lady Rosetta, respectively. These data suggest that much more Cd was lost from the stem of Lady Rosetta, compared with that from Cara. By assuming all net loss of Cd was translocated to the tuber, loss from stem and leaf contributed 66% and 69% of the final tuber Cd in Cara and Lady Rosetta, respectively. According these results, remobilization of Cd from vegetative organs was the major source of tuber Cd, though the two cultivars did not exhibit any marked difference in this phenomenon.

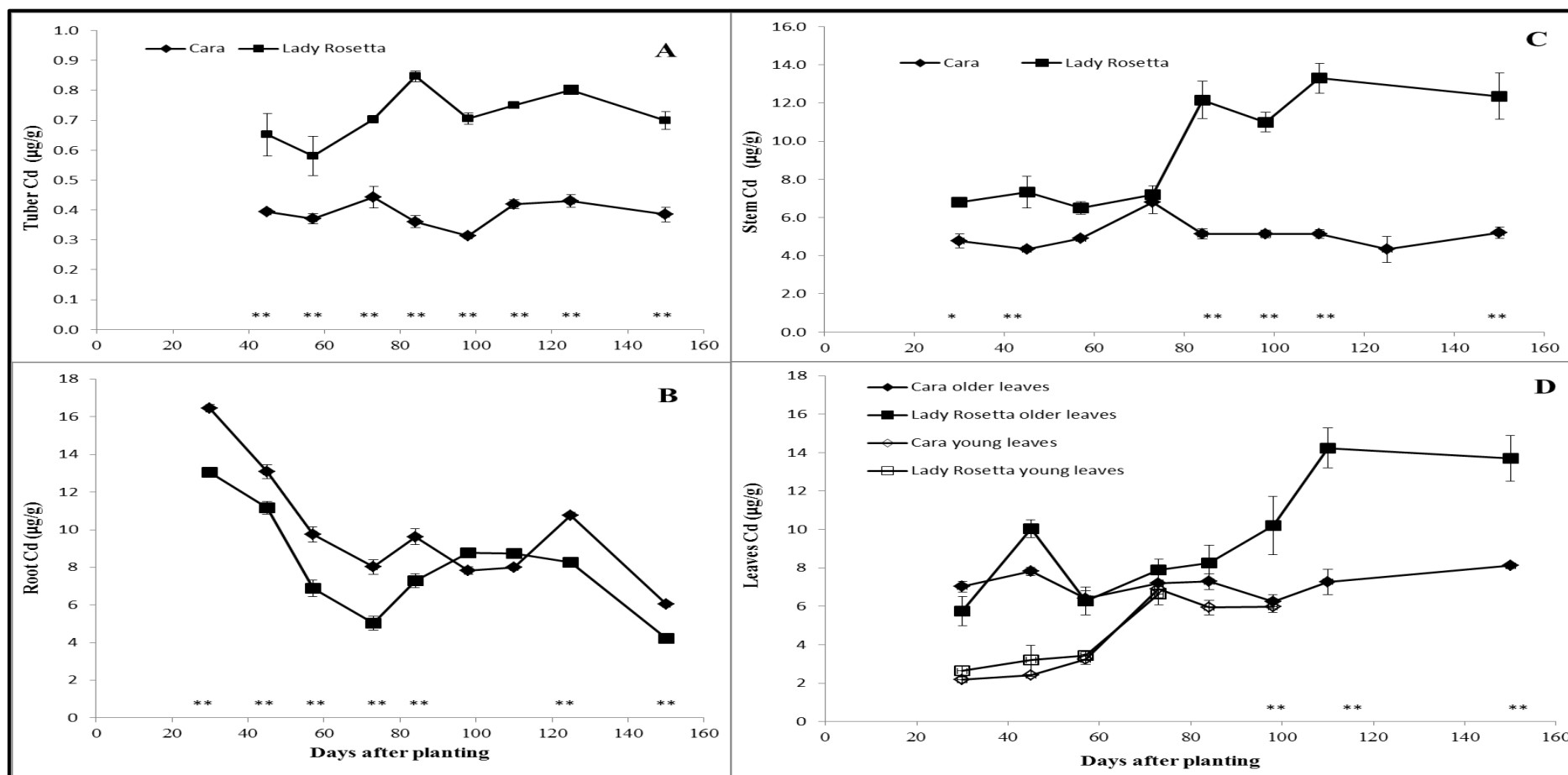


Figure 3.3 Cd concentrations in different organs of the two cultivars over time. Data points represent the mean \pm standard error of the mean (n=3).

** Significant ($P < 0.01$) difference between the two cultivars at a given harvest time. In figure D, statistical test presented for older leaves only.

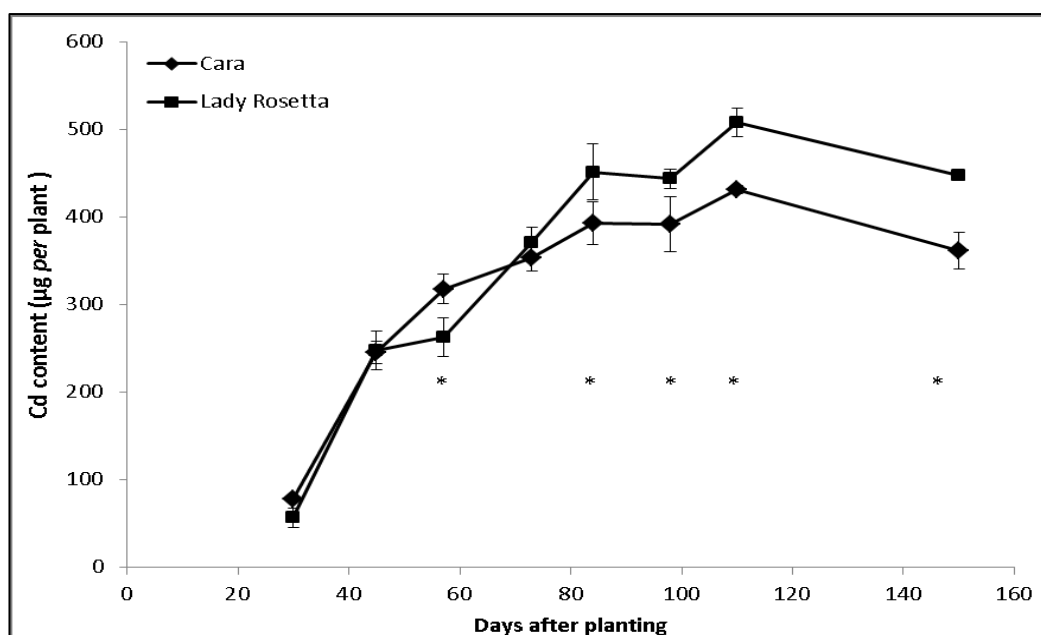


Figure 3.4 Cd accumulation during growth of the two cultivars from 30 DAP to final harvest. Data points are the mean value \pm standard error of the mean ($n=3$); * Significant difference ($P<0.05$) between the Cd content of the two cultivars at the correspondent DAP.

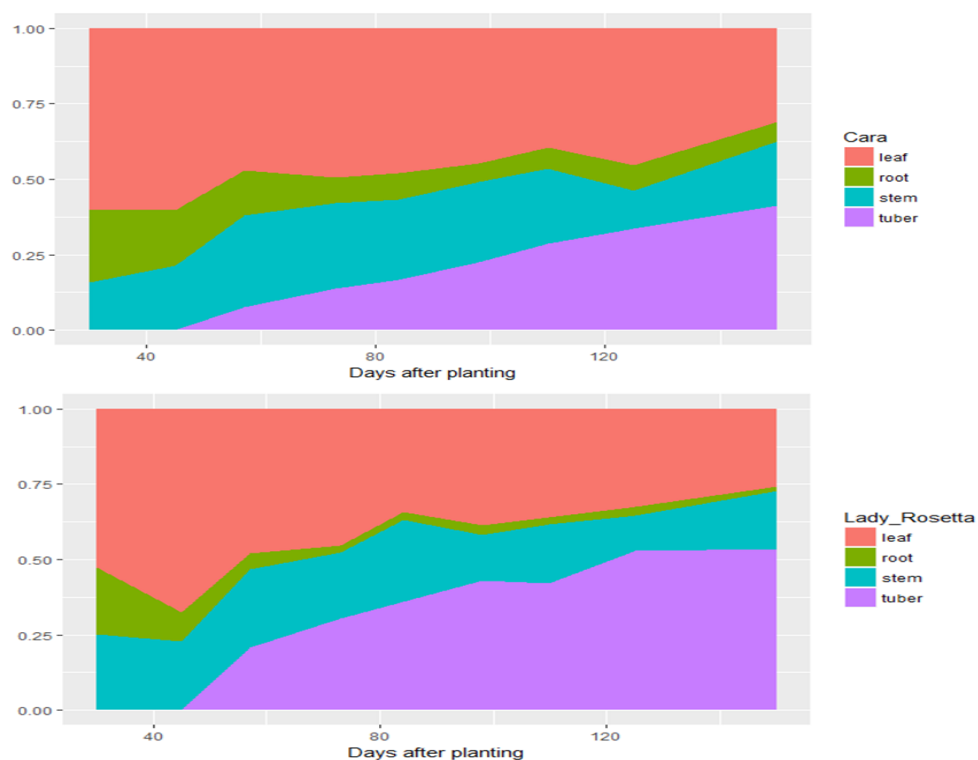


Figure 3.5 Distribution of Cd in all organs of potato plants over the growth period.

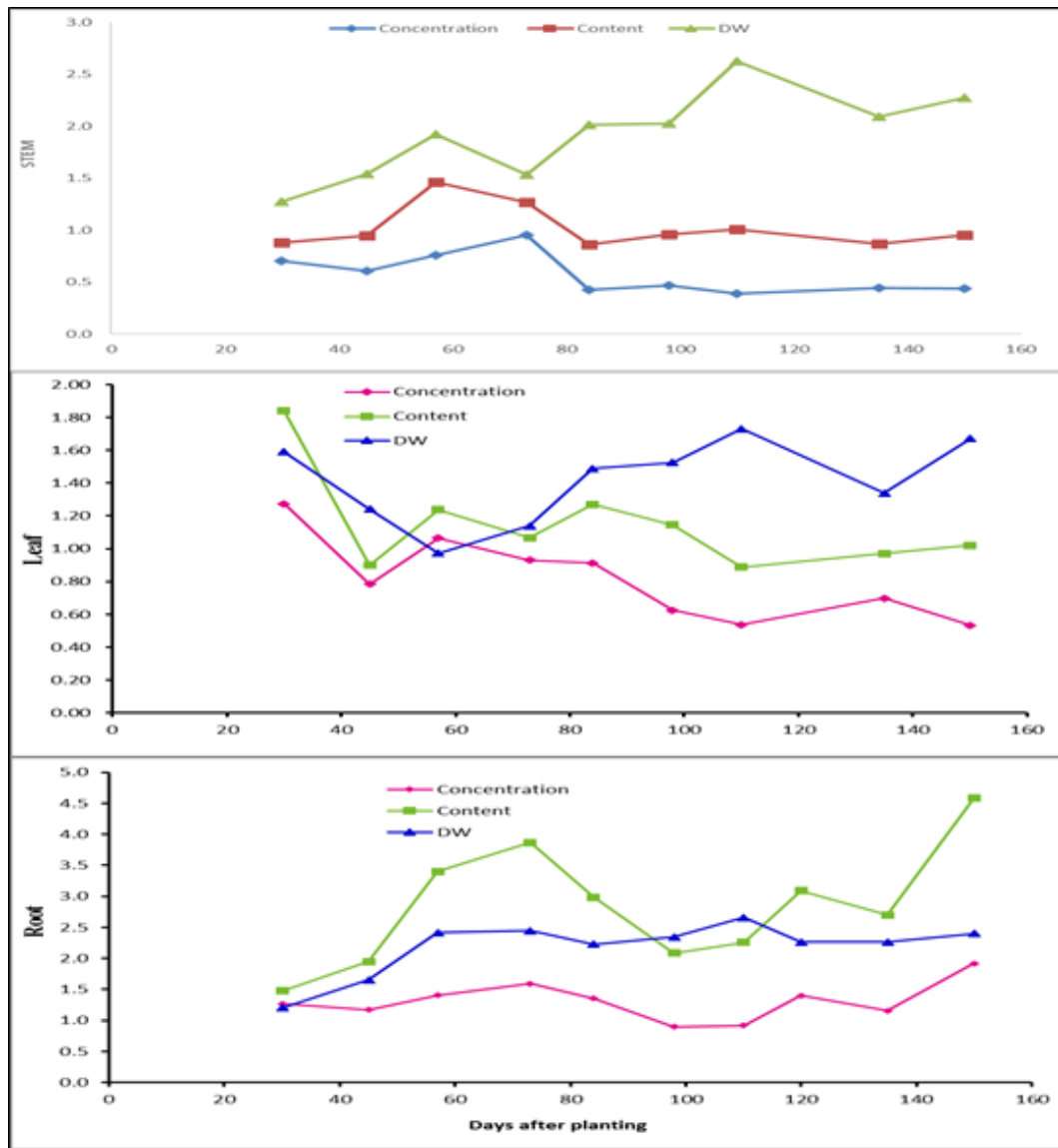


Figure 3.6 Evaluating the effects of DW variation between the two cultivars on Cd concentration and Cd content in different organs (leaf, stem and root) with time as measured by the ratios of these traits. For each organ, ratios of DW, Cd-content and Cd-concentration were calculated by dividing Cara by Lady Rosetta values. Cd-content values in each organ were determined by multiplying the biomass (g DW) by Cd-concentration ($\mu\text{g/g}$). Box plots display the full range of variation (n=3).

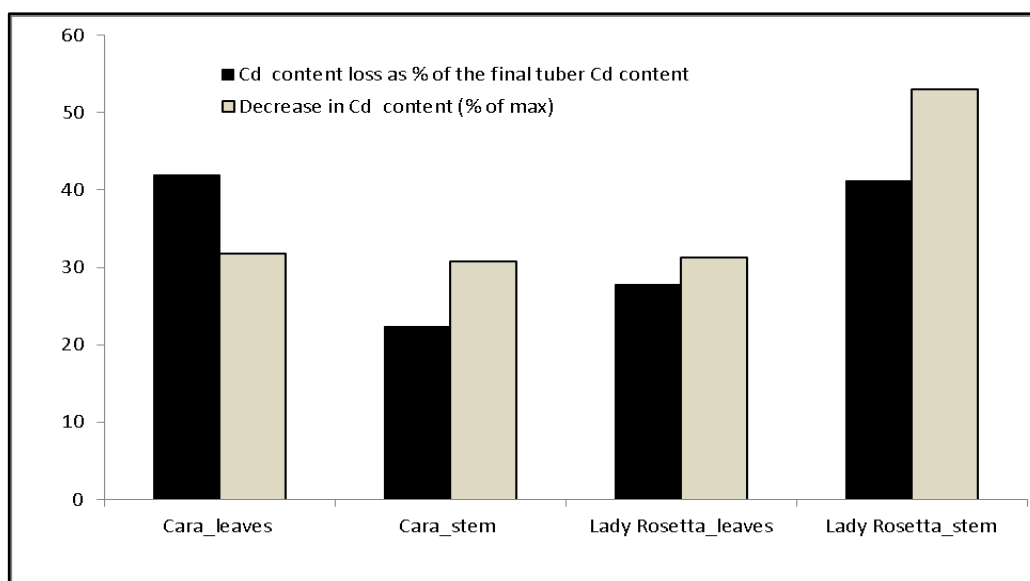


Figure 3.7 Percentage loss of Cd content (% of maximum content) from leaves and stems and loss of Cd from leaves and stems expressed as % of the final tuber Cd content.

3.3.3. Zn uptake and distribution between the organs of two cultivars

3.3.3.1. Zn concentration between organs of the two cultivars over time

Tuber Zn concentration was significantly affected by cultivar ($P < 0.01$), time ($P < 0.01$) and the interaction between the two factors ($P < 0.05$) (Appendix-Table 8.6). For root Zn concentration, no significant differences were observed between cultivars, nor was the interaction between cultivar and time significant. However, root Zn concentration significantly ($P < 0.01$) declined with time. Stem and leaf Zn concentration showed significant ($P < 0.01$) differences with respect to cultivar and time, but no significant interaction between cultivars and time was observed (Appendix-Table 8.6).

Changes in Zn concentration between organs of the two cultivars are presented in Figure 3.8. At 45 DAP, tuber Zn concentration was larger when the tubers were smaller for both cultivars. Then, it sharply declined until 57 DAP and remained constant for the rest of the growth period. Lady Rosetta had significantly ($P < 0.01$) greater tuber Zn concentration than Cara at 84 DAP (Figure 3.8A). Patterns of root Zn concentration between the two cultivars were similar. The root Zn concentration showed a slight decrease as the growth period progressed (Figure 3.8B).

The pattern of change in leaf Zn concentrations was somewhat similar to changes in tuber Zn concentration, being greater in the first 45 DAP, after which a sharp decline occurred until 57 DAP, with no major changes thereafter. Lady Rosetta had significantly ($P < 0.01$) greater leaf Zn concentration than did Cara, beginning from 73 days of planting (Figure 3.8C).

Stem Zn concentration did not consistently increase or decrease over the growth period but fluctuated over much of the growth period (Figure 3.8). Surprisingly, Zn concentrations were similar at early and late growth stages for both cultivars. Lady Rosetta had significantly ($P < 0.01$) greater Zn concentrations in stems than did Cara throughout the growth period, except at 73 DAP (Figure 3.8D). From these results, the important observations are:

- (i) Leaf and tuber Zn concentration exhibited similar patterns over time,
- (ii) Stem Zn concentration was the only significant difference between the two cultivars during the first 57 DAP, with that difference continuing throughout the growth period, and
- (iii) Regardless of cultivar, the concentration of Zn in organs followed the order: stem > leaf \geq root > tuber Zn.

3.3.3.2. Total Zn uptake and organ distribution of Zn

At final harvest, there was no difference in total Zn uptake (per plant) between the two cultivars (Table 3.3, Appendix-Table 8.1). There was a considerable increase in Zn content until 100 and 110 DAP for Lady Rosetta and Cara, respectively (Figure 3.9).

Similarly, the overall distribution of Zn between organs at final harvest showed that the two cultivars differed in partitioning of Zn to stem, tuber and root. However, leaf Zn content was similar between the two cultivars (Table 3.3). Lady Rosetta accumulated more Zn in the tubers than did Cara. However, Cara retained more Zn in the stems and roots than did Lady Rosetta. Despite differences between the cultivars with respect to root Zn content, the contribution of root Zn content to the total plant Zn in both cultivars was negligible. Therefore, the main difference between the two cultivars was Zn partitioning between stems and tubers (Table 3.3).

Patterns of Zn partitioning within organs of the two cultivars are presented in Figure 3.10. At the first harvest, 30 days after planting, the overall distribution of Zn in the

organs of the two cultivars was allocated as follows: 42% to leaves, 47% to stem and 11% to roots of Lady Rosetta, whereas Cara allocated 49% to leaves, 41% to stem and 10% to roots.

At final harvest, 77 and 68% of the total plant Zn was found in the tubers of Lady Rosetta and Cara, respectively. By contrast, stems of Lady Rosetta and Cara retained 17% and 25% of total Zn, respectively. Both cultivars retained approximately the same amount of Zn in the leaves (5%) (Table 3.3, Figure 3.10), indicating effective remobilization of leaf Zn to the tubers or other organs. As noted above, little Zn was retained in roots of either cultivars, indicating that root-to-shoot translocation was effective (Table 3.3).

Since the two cultivars differed in dry weight accumulation over time, it was important to evaluate the effect of dry weight on Zn concentration and Zn content among organs of the two cultivars. The comparisons were performed by taking the ratio of changes in dry weight, Zn concentration and Zn content between the two cultivars over time. As shown in Figure 3.11, the changes in dry weight were different between the two cultivars, with Cara accumulating greater DW values in stem, leaf and root than did Lady Rosetta. On the other hand, the changes in Zn concentration were somewhat similar between the two cultivars for both stem and root, with a slight decrease in Zn concentration of Cara, compared to Lady Rosetta, over time. Unlike Cd (Figure 3.6), Zn concentration among organs was not influenced by the differences in dry weight accumulation between the two cultivars, suggesting that Zn is a highly regulated essential element for plant growth and development.

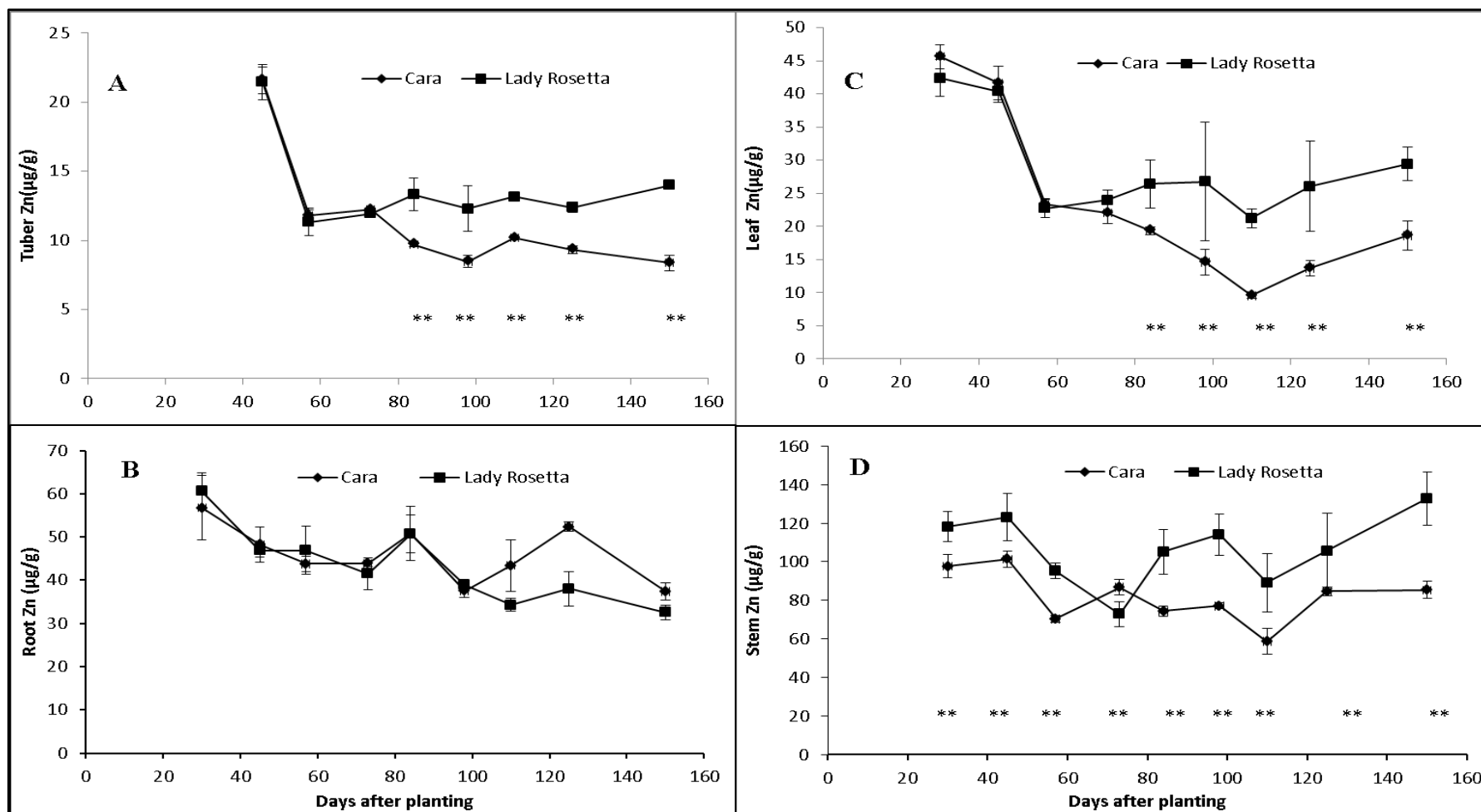


Figure 3.8 Concentrations of Zn in different organs of the two cultivars over time. Data points represent the mean \pm standard error of the mean (n=3). ** Significant ($P \leq 0.01$) difference between cultivars at a given harvest point.

Table 3.3 DW, Cd and Zn concentration and content of different organs of the two cultivars at final harvest

Organ	Cultivars	DW (g)	Diff	[Cd] (µg/g)	Diff	Cd content (µg)	Diff	Cd content (% of the total plant Cd)	[Zn] (µg/g)	Diff	Zn content (µg)	Diff	Zn content (% of total plant Zn)
Tuber	Cara	371	61**	0.4	-0.3**	143	-	39	8	5.61	3209	677**	68
	Lady Rosetta	310		0.7		216	73**	50	14	**	3886		77
Root	Cara	3	2**	6	2**	18	13**	5	37	4ns	111	70**	1
	Lady Rosetta	1		4		6		1	33		41		2
Stem	Cara	14	7**	5	-7**	72	-7ns	36	19	10**	253	17ns	5
	Lady Rosetta	7		12		79		31	29		270		5
Leaves	Cara	14	4*	8	-6**	129	-3ns	20	85	48**	1180	302**	25
	Lady Rosetta	10		14		132		18	133		877		17
Whole plant	Cara	402	74*	-	-	362	-86*	-			4752	323ns	
	Lady Rosetta	328		-		448					5074		

**, * Significant at $P < 0.01$ and $P < 0.05$, respectively, ns = non-significant difference, Diff = difference value when Cara subtracted from Lady Rosetta at final harvest, DW = dry weight *per* plant. Each value is the mean of three plants.

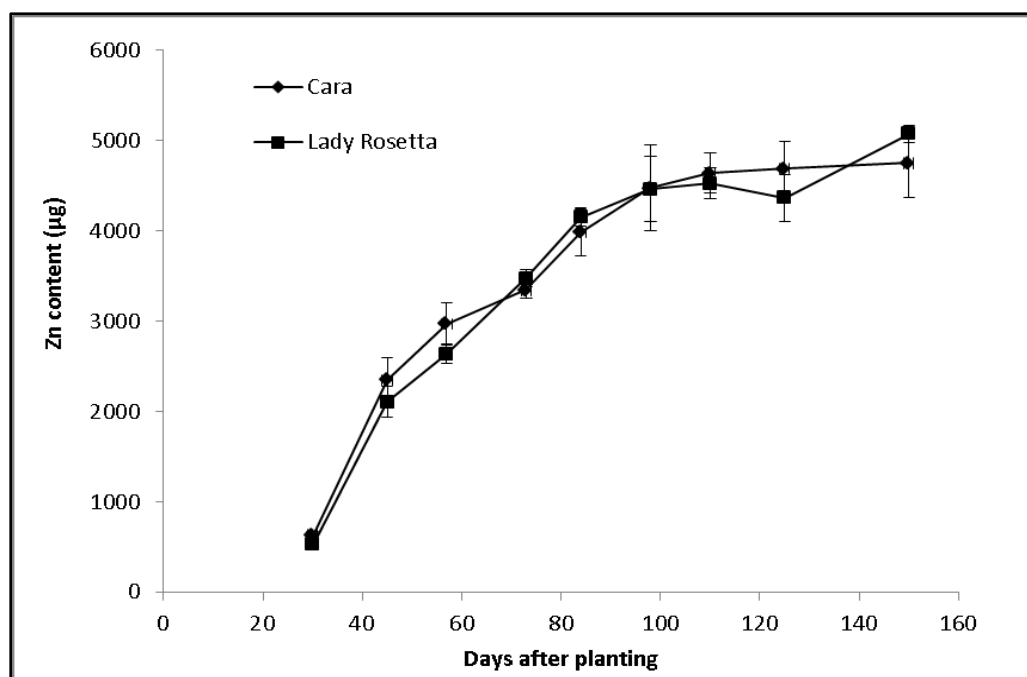


Figure 3.9 Overall Zn accumulation in potato plants over the growth period. Values represent the mean of 3 plants and + standard error of the mean.

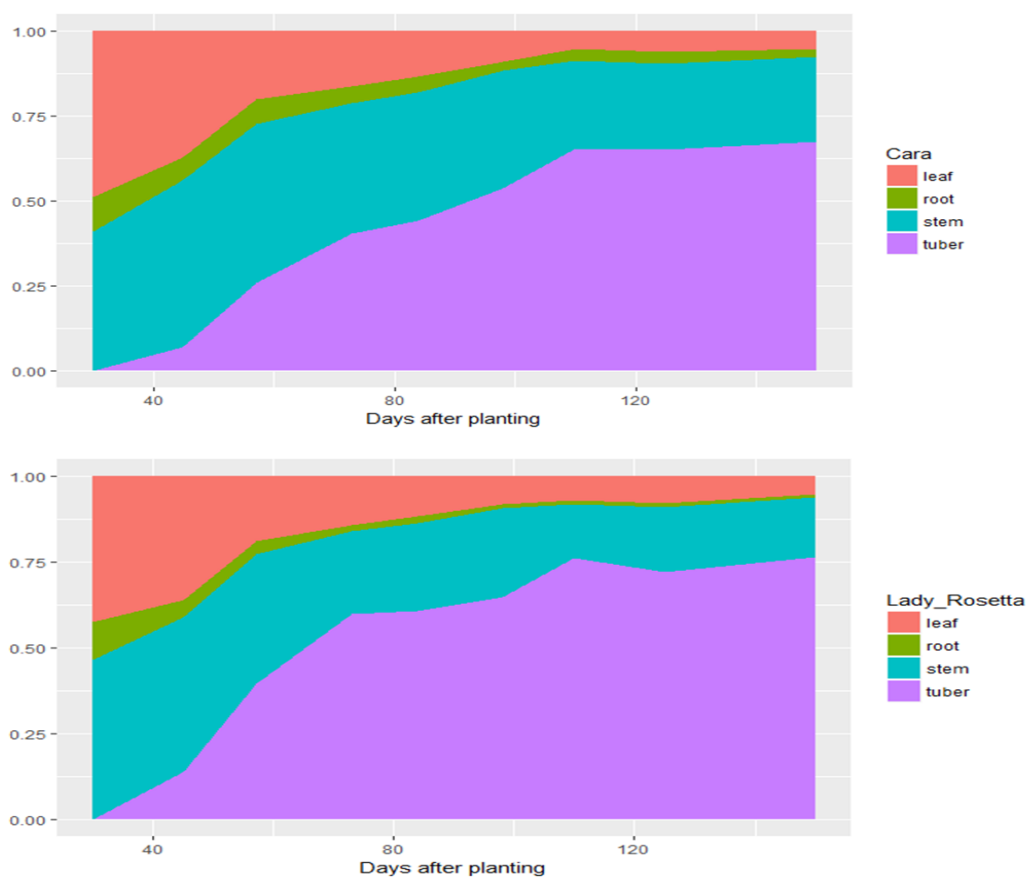


Figure 3. 10 Distribution of Zn between organs of the two cultivars over time.

3.3.3.3. Remobilization of Zn

Remobilization (net loss of Zn) from vegetative organs (stems and leaves) in the two cultivars is shown in Figure 3.12. Net loss of leaf Zn, calculated as final Zn content expressed as a percentage of the maximum Zn content in that organ, was approximately 72% and 64% for Cara and Lady Rosetta, respectively. Similarly, net loss combined from stems was approximately 24% for each cultivar. Compared to the amount of Zn present in tubers at final harvest, loss of Zn from leaves and stems only accounted for 33% and 20% of the final tuber Zn in Cara and Lady Rosetta, respectively.

The experiment was repeated at selected time points. The patterns organs of dry weight accumulation, Cd concentration and Cd partitioning were similar that of the first-year experiment (Appendix-Figures 8.1, 8.2, 8.3). Similarly, the patterns of Zn concentration and Zn partitioning between organs was similar with that of the first-year experiment (Appendix-Figures 8.4, 8.5). Furthermore, the two cultivars showed consistent differences with respect to total dry weight and whole-plant Cd uptake but not for whole-plant Zn content (Appendix-Figure 8.6). The patterns of growth and whole-plant Cd/Zn concentration were also similar to that of the first year (Appendix-Figure 8.7). In general, the second-year experiment confirmed that differences between the two cultivars in terms of Cd uptake (but not Zn), biomass and concentration were consistent over the years.

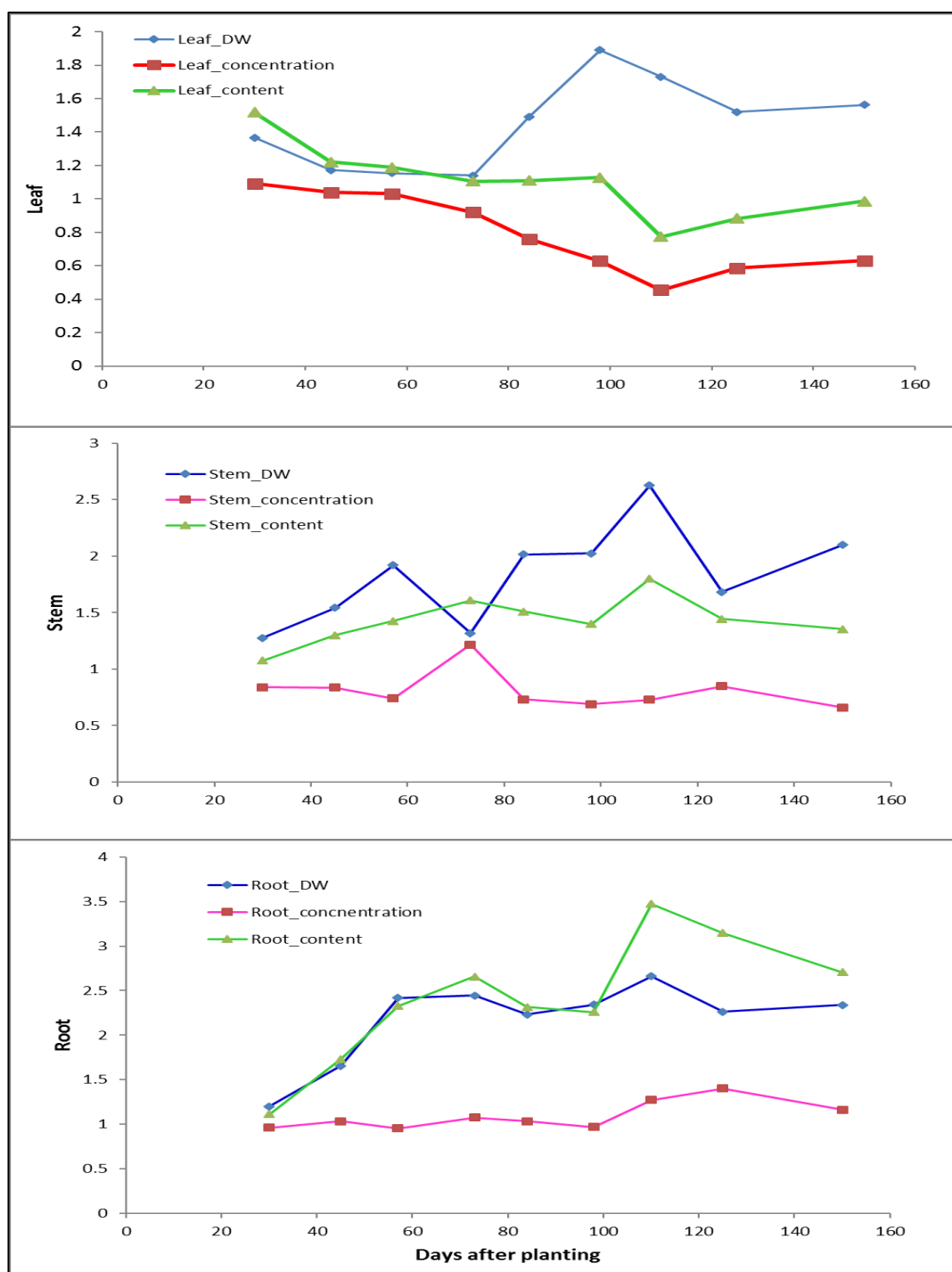


Figure 3.11 Effects of DW variation between the two cultivars on Zn concentration and Zn content in different organs (leaf, stem and root) with time as measured by the ratios of these traits. For each organ, ratios of DW, Zn-concentration and Zn-content were calculated by dividing Cara values by Lady Rosetta values. Cd-content values in each organ were determined by multiplying the biomass (g DW) by Cd-concentration ($\mu\text{g/g}$). Box plots display the full range of variation ($n=3$).



Figure 3.12 Percentage loss of Zn content (% of maximum content) from leaves and stems and loss of Zn from leaves and stems expressed as % of the final tuber Zn content.

3.4. Discussion

This study clearly established that the two cultivars differed in total Cd uptake and distribution between organs. However, the differences in tuber Zn concentration were associated with internal distribution of Zn rather than to differences in total Zn uptake. Furthermore, dry weight accumulation affected the distribution of Cd and Zn. The study also suggested that remobilization of Cd from vegetative organs (stem and leaf) are the main source of tuber Cd.

3.4.1. Growth and Cd/Zn dynamics

Dynamics of element concentrations including Cd and Zn can be influenced by the growth rate of organs (Marschner, 1995; Waters & Grusak, 2008; Impa *et al.*, 2013). In this study, Cd and Zn concentrations declined in all organs over the first 57 DAP (Figure 3.3, Figure 3.8) while plants were growing actively (Figure 3.2). As shown in Figure 3.13, Lady Rosetta had slightly larger weight-normalized Cd and Zn concentrations than Cara 84 days after planting. This difference is most likely related to the continued growth of Cara relative to Lady Rosetta, resulting in growth dilution of the element concentrations. Similar results have been reported in *A. thaliana*

(Waters & Grusak, 2008) and rice (Impa *et al.*, 2013) where growth affected element concentrations.

From these data (Figures 3.3, 3.8, 3.13), it is possible that growth habit or difference in dry weight accumulation of cultivars may influence organ Cd and Zn concentration through dilution. In line with this, the trends in tuber Zn concentration showed a slight decline with growth regardless of the cultivars being tested, suggesting dilution effects on tuber Zn concentration. These results are consistent with the view that tuber yield affected tuber Zn concentration through dilution (Subramanian, 2012). However, a previous study did not show any significant correlation between tuber yield and tuber Zn concentration (White *et al.*, 2009). With respect to Cd, tuber Cd varied less over time in both cultivars (Figure 3.3A), indicating that Cd dilution associated with tuber yield is minor.

Differences in tuber Cd and Zn concentrations between the cultivars appeared at the same growth stage as differences in leaf Cd and Zn concentrations. Greater leaf Cd and Zn concentrations in Lady Rosetta than Cara were matched with larger tuber Cd and Zn concentrations. Similar results were reported by Jönsson and Asp (2011), that a cultivar with greater leaf Cd had greater tuber Cd than a cultivar with less leaf Cd. Kromann *et al.* (2016) also found similar results that larger shoot Zn concentration corresponded to larger tuber Zn concentration. At final harvest, the two cultivars had similar levels of total Cd content, despite Cara having 72% greater non-tuber organ dry weight compared to Lady Rosetta (Table 3.3). Similarly, the non-tuber Cd concentration of Lady Rosetta was 72% greater than Cara.

3.4.2. The cultivars differed in total Cd uptake but not in total Zn uptake

Significant ($P < 0.01$) difference was observed for plant Cd uptake between the two cultivars. Lady Rosetta taking up more than 20% more Cd than Cara. To date, this is the first report concerning potato cultivar differences in plant Cd uptake. Variations in Cd uptake have also been reported in wheat (Kubo *et al.*, 2011; Arduini *et al.*, 2014; Perrier *et al.*, 2016), soybean (Arao *et al.*, 2003), sunflower (Laporte *et al.*, 2015), hot pepper (Xin *et al.*, 2015), tomato (Zhu *et al.*, 2011) and pak choi (Xia *et al.*, 2016). However, a study by Dunbar *et al.* (2003) reported that the difference in tuber Cd accumulation between two Australian potato cultivars was due solely to altered

partitioning of total Cd between plant organs, and that the cultivars did not differ in total Cd uptake. These differences are most likely related to the cultivars under study ('Kennebec' and 'Wilwash'), as potato has wide genetic diversity coupled with geographical adaptation (Bradshaw *et al.*, 2006), which may result in a range of different physiological mechanisms of uptake of Cd. Furthermore, differences in experimental conditions could also influence the results, as the study by Dunbar *et al.* (2003) was conducted in sand supplemented with 1 L/day of 10 nM Cd solution, while the current study was done in natural soil with a relatively increased Cd concentration (Table 3.1). According to Arduini *et al.* (2014), differences in Cd uptake by durum wheat cultivars were dependent on the level of Cd in the soil. In a low-Cd soil, the two durum wheat cultivars studied did not show differences in Cd uptake while clear differences were observed as the Cd supply in the soil increased.

Differences in Cd uptake can arise from difference in shoot biomass differences or root-related traits (Lu *et al.*, 2013; Laporte *et al.*, 2015; Kubo *et al.*, 2016; Xia *et al.*, 2016). However, plant biomass does not explain the differences in Cd uptake in this experiment, as Lady Rosetta accumulated more Cd despite producing less biomass than Cara (Figures 3.1 and Figure 3.4). A recent study in wheat also showed that Cd uptake was not associated with total dry weight accumulation (Kubo *et al.*, 2016).

Further analyses also showed that root biomass and root length did not show any association with Cd uptake as Cara took up less Cd despite producing larger root biomass and longer roots (Figures 3.2B and 3.4). Similar results have been reported in other crops, such as sunflower (Laporte *et al.*, 2015), rice (Kubo *et al.*, 2011) and peanut (Lu *et al.*, 2013). So, if root biomass was not a driving force for root Cd uptake, what other root trait(s) are important for root Cd uptake? Previous studies examining the relationship between root systems and nutrient uptake (Fitter *et al.*, 1991; Kubo *et al.*, 2011; Lu *et al.*, 2013; Xia *et al.*, 2016) found that the volume of the root (root surface area) and the number of root tips are important traits which drive nutrient acquisition from soil. Kubo *et al.* (2011) also reported that a wheat variety with more root branching had larger whole-plant Cd content than that of the variety with a less root branching, and that root frequency (root number cm⁻² soil surface) had a significant positive correlation with Cd quantity in whole-plant organs. In agreement with those studies, the high-accumulating cultivar absorbed 250% more Cd per g root compared to the low-accumulating cultivar at final harvest. Furthermore, the two

cultivars also differed with respect to root system, as Lady Rosetta exhibited a relatively short seminal root, with more branching, while Cara developed long roots, with relatively little branching, compared to Lady Rosetta (personal observation).

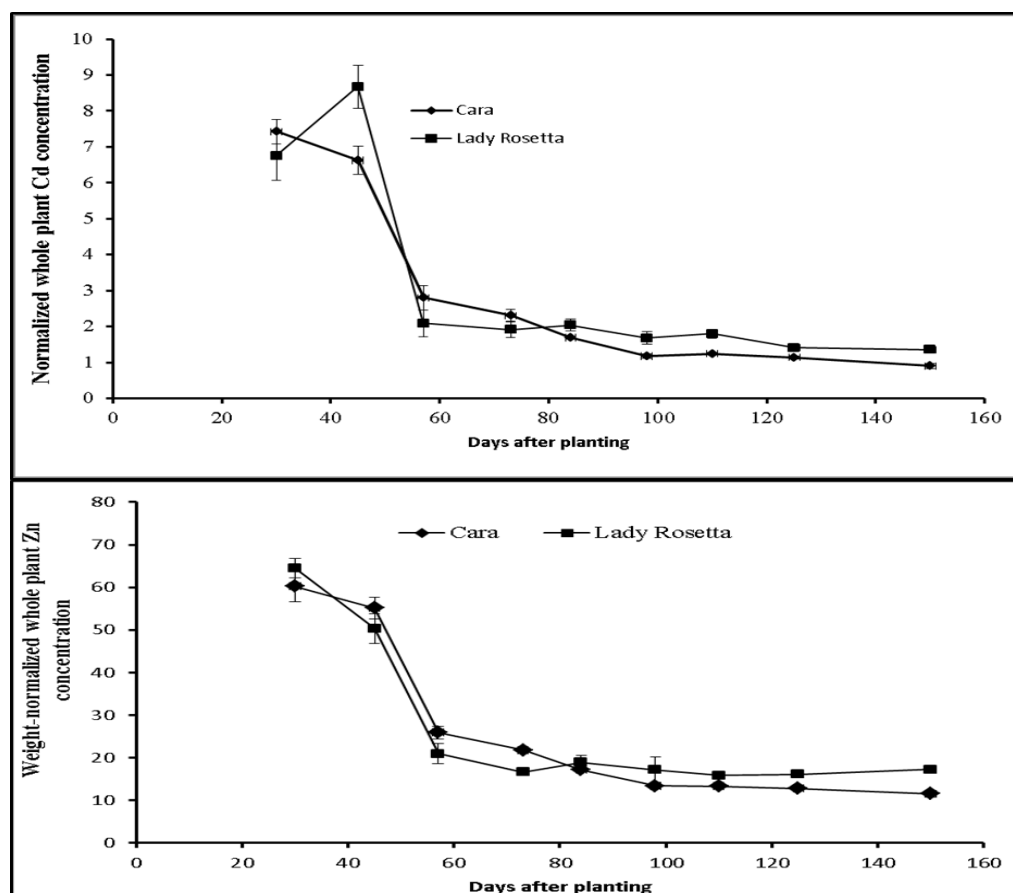


Figure 3.13 Weight-normalized whole plant Zn concentrations ($\mu\text{g/g}$) between the two cultivars with growth. Values are means of three plants ($n=3$).

3.4.3. Root-to-shoot translocation of Cd and Zn

Root-to-shoot translocation is a determinant factor for tuber Cd concentration in potato (Dunbar *et al.*, 2003) and other Solanaceae species (Mori *et al.*, 2009). As only minor amounts of Cd were absorbed by the tubers and their associated stolons and stolon roots, as demonstrated by Reid *et al.* (2003), the root-to-shoot translocation ratio was calculated by dividing the total Cd content of shoot and tuber by root Cd content. The root-to-shoot translocation of Cd was greater in Lady Rosetta than in Cara, as shown by the consistently larger ratio in Figure 3.14A. Differences in root-to-shoot translocation of Cd have been attributed to differences in root sequestration and/ or in xylem loading between cultivars (Mori *et al.*, 2009). Similarly, Zn root to shoot

translocation was more in Lady Rosetta than Cara (Figure 3.14B). Similarly, Zn root-to-shoot translocation was greater in Lady Rosetta than Cara (Figure 3.14B). In this study, the contributions of root Cd sequestration to the total plant Cd and the amount of Zn allocated to the root were minor fractions, and differences were mainly accounted for by differences in root biomass between cultivars. Therefore, the root-to-shoot translocation appears not to be a major impediment for uptake and translocation of Cd and Zn to the above-ground organs of the potato plant.

3.4.4. Cd and Zn partitioning between shoot and tubers

At final harvest, the two cultivars exhibited differences in partitioning of Cd (Figure 3.5). In Lady Rosetta, 50% of the total Cd was allocated to the tubers while other 50% remained in non-tuber organs. On the other hand, only 39% of the total Cd was allocated to the tubers of Cara. Despite greater total Cd uptake by Lady Rosetta, this cultivar also allocated much more of the Cd taken up to the tubers, at least 51% greater than Cara. The question is, why did Lady Rosetta translocate much more Cd than Cara? According to this study and evidence from other crops (Kubo *et al.*, 2008; Arduini *et al.*, 2014; Xin *et al.*, 2015; Perrier *et al.*, 2016), shoot biomass affects shoot Cd concentration through dilution and these differences in shoot Cd concentration may create differences in gradient between the sources and the phloem, and offer difference in ability to load Cd into the phloem. This hypothesis is strengthened by the fact that remobilization of Cd from the shoot is the major source of tuber Cd, as 66% and 69% of the total tuber Cd came from remobilization in Cara and Lady Rosetta, respectively. These results are consistent with studies in different crops, such as wheat (Hart *et al.*, 1998; Harris & Taylor, 2001), rice (Tanaka *et al.*, 2007; Kashiwagi *et al.*, 2009; Rodda *et al.*, 2011) and sweet potato (Huang *et al.*, 2015) that remobilization is the main source of Cd translocated to the edible portions.

Normalized partitioning of an element within a plant provides information about the difference between the two cultivars and between organs within cultivars over time, regardless of plant size (Waters & Grusak, 2008; Sperotto, 2013). In terms of PQ, a larger PQ value was recorded in the leaf than other organs in both cultivars, indicating that Cd may be transported through the transpiration stream, and stored in the leaf (Figure 3.15).

Regardless of cultivar differences, the concentration of Cd in the tuber was very low compared to that in other organs (stems, leaves and roots) (Table 3.3). A similar observation was reported by Chen *et al.* (2014), with leaves exhibiting Cd concentrations more than 15-fold more than tubers. The question is, why does this much Cd concentrate in the shoot as opposed to the tuber, and what prevents its transport to the tubers? Reid *et al.* (2003) found that Cd is mobile in phloem tissues, a finding which may be associated with the higher affinity of Cd for sulphhydryl groups, which present as side groups of proteins. The binding of Cd with sulphhydryl groups on the sieve tubes of phloem could permit the greater mobility of Cd via the phloem pathway. Furthermore, studies in wheat (Hart *et al.*, 1998) and rice (Tanaka *et al.*, 2007) indirectly confirmed Cd mobility in phloem since they observed that the amount of Cd in the phloem was positively correlated to the amount of Cd in the grain. If Cd is mobile in the phloem, what other factors regulate differences in Cd concentration between shoot and tuber? The most likely reason for the difference in Cd concentration between those tissues is that Cd may be sequestered by high-molecular-weight complexing agents in the vacuoles of leaves and deposited in the cell wall-binding sites of the stems (Gallego *et al.*, 2012; Xu *et al.*, 2013; Chen *et al.*, 2014). Scanning electron microscopic (SEM) examinations of Cd in the leaves and stems of potato indicated that Cd was mainly localized in the cortex and adjacent phloem of the stem and in the spongy and palisade mesophyll of the leaf (Xu *et al.*, 2013; Chen *et al.*, 2014).

Zn partitioning between the stems and the tubers was the main difference in Zn dynamics between the two cultivars examined in this study. Cara contained more Zn in the stems, whereas Lady Rosetta accumulated more Zn in the tubers (Figure 3.10, Table 3.3). The question is, why did Cara have greater Zn in the stem than Lady Rosetta? Two hypotheses can be suggested: preferential retention of Zn in the stems of Cara and/or dry weight-driven accumulation, as Cara had larger stem dry weight than Lady Rosetta. The PQ can estimate Zn partitioning between organs of the plant, regardless of dry weight. The PQ values (Figure 3.175) clearly showed that stem had larger PQ values compared to other organs in both cultivars, indicating that relatively more Zn was partitioned to the stem as the plant grew. Surprisingly, the two cultivars had similar PQ values and Zn accumulation patterns in the stem over time, indicating that the greater Zn accumulation in Cara than in Lady Rosetta was not associated with

preferential distribution of Zn in the stems. However, the larger stem Zn accumulation in Cara is most likely favoured by the greater stem dry weight accumulation in this cultivar, compared to Lady Rosetta. On the other hand, tubers of both cultivars had the least PQ values compared to other organs, and these values were largely constant over time (Figure 3.15). These results are consistent with previous reports by Kromann *et al.* (2016) and White *et al.* (2017), that the rate of Zn partitioning to tubers was very small compared to that of shoots of potato from both soil and foliar application, suggesting that Zn mobility in the phloem of potato can be restricted or slow.

Despite greater stem Zn content compared to overall Zn accumulation in both cultivars, net loss of Zn content from the stem was smaller (Figure 3.10). The question is, why did the stem retain more Zn content than the leaves? These results suggest that Zn may be deposited in the cell wall and intercellular spaces of stems. Studies in related solanaceous species, *Solanum nigrum* and *Solanum lycopersicum*, found that Zn is mainly deposited in the intercellular spaces and on the cell wall (Marques *et al.*, 2007; Muschitz *et al.*, 2009; Samardjieva *et al.*, 2015). Studies in cereal crops, wheat (Garnett and Graham 2005) and rice (Jiang *et al.*, 2008; Yoneyama *et al.*, 2010; Yamaguchi *et al.*, 2012; Yamaji & Ma, 2014) also showed that the stem is a store of Zn that can be utilized when Zn uptake by roots is low and may be the main site of Zn loading into the phloem. Moreover, reports in graminaceous plants, such as rice and barley, have emphasized that stem nodes play a key role in preferential distribution and storage Zn (Yamaji & Ma, 2014).

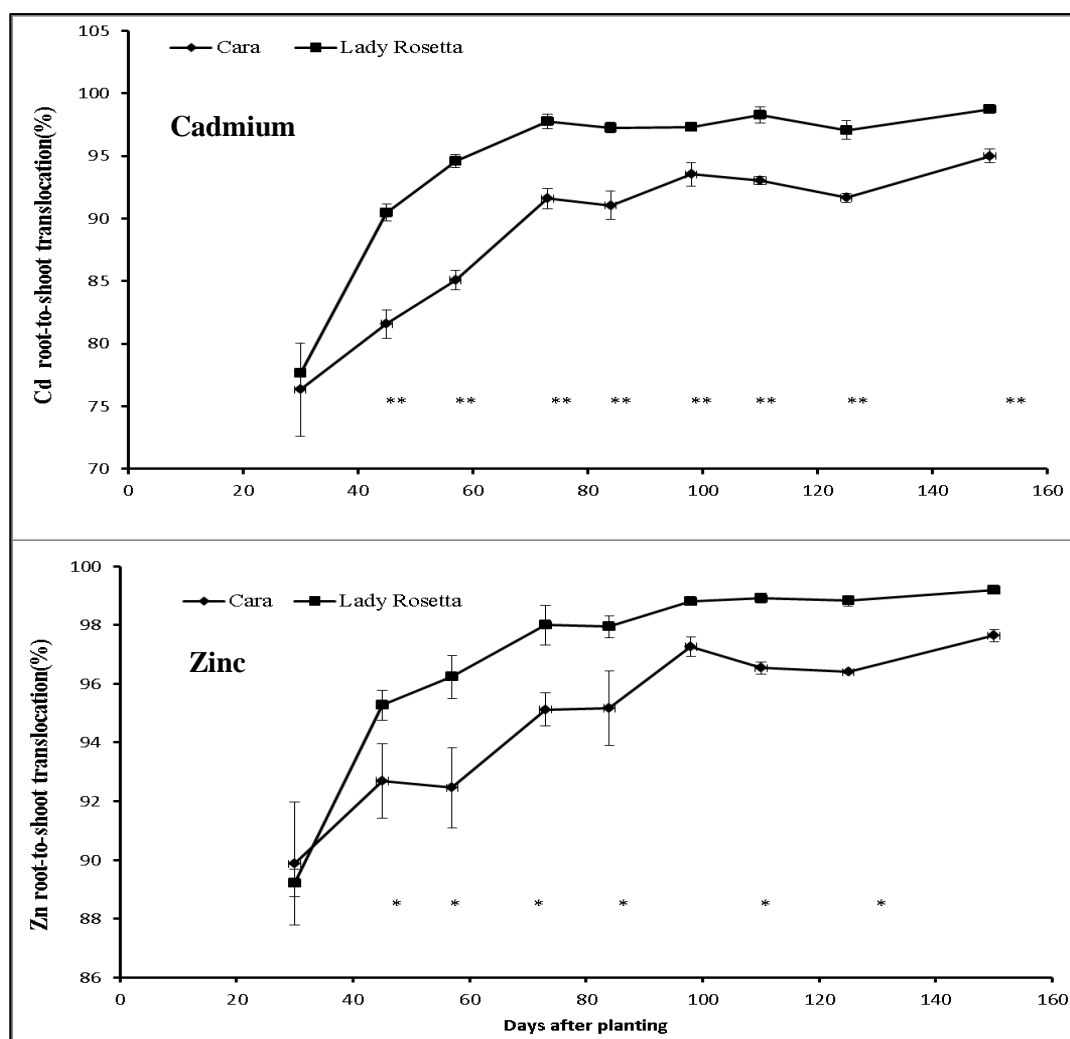


Figure 3.14 Translocation of Cd and Zn to the shoot over time for the two cultivars. Cd/Zn translocation was calculated as the amount of Cd/Zn in shoots and tubers divided by the total whole-plant Cd. Data points are mean \pm standard error of the mean (n=3). ** Significant (P<0.01) difference between the cultivars at a given harvest time.

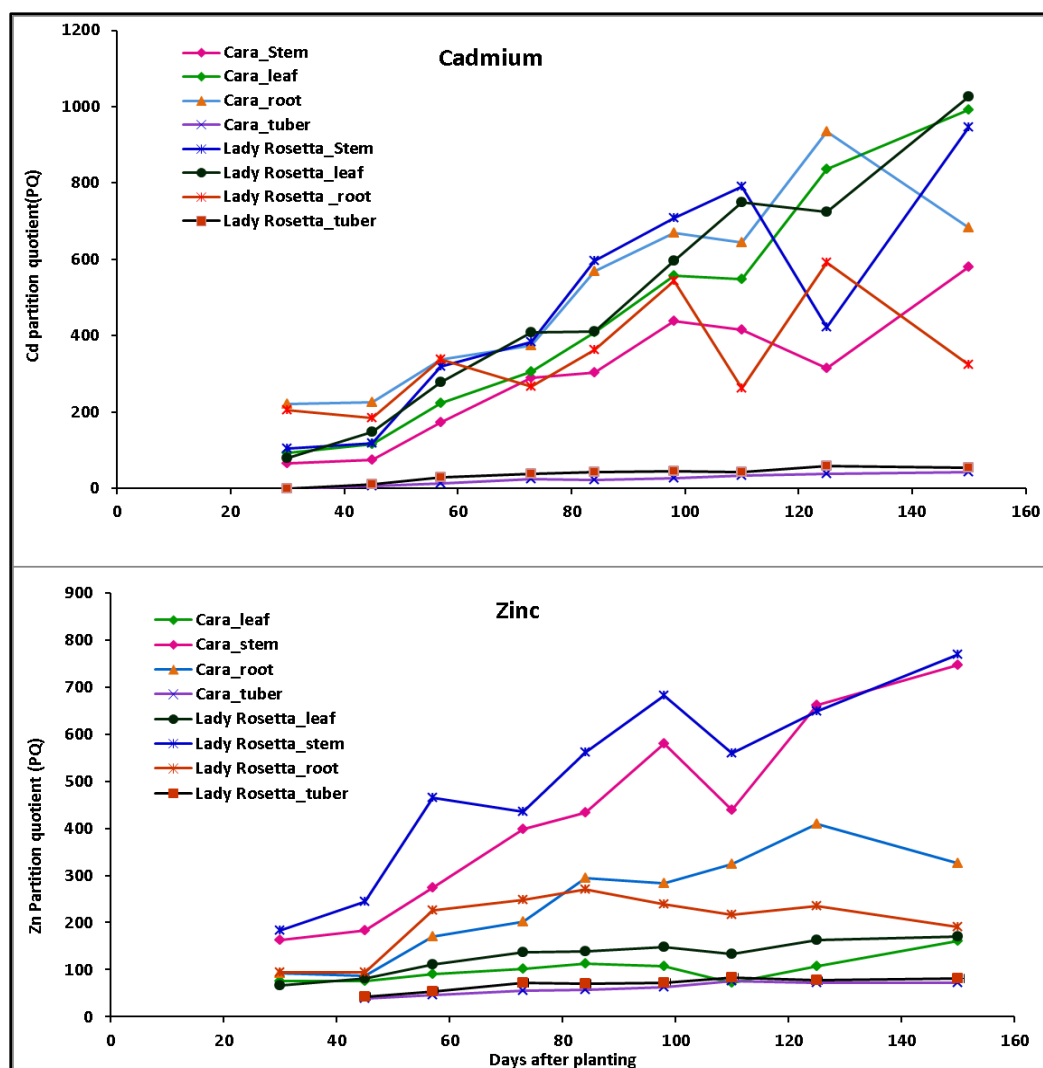


Figure 3.15 Partitioning quotient values for Cd and Zn in different organs of the two cultivars over time.

To summarize, the results revealed that differences in tuber Cd accumulation are driven by a three-step process: Cd uptake, root-to-shoot translocation and partitioning of Cd between shoot and tuber. Cd uptake was greater in the cultivar with greater tuber Cd (Lady Rosetta), though the underlying mechanism has not been determined. Root-to-shoot translocation was very efficient in both cultivars with slightly larger translocation in Lady Rosetta than in Cara. Shoot-to-tuber translocation was considerably less efficient, with a significant Cd quantity remaining in the shoot at harvest. Nevertheless, the cultivars exhibited differences in both shoot-Cd concentration (with the greater tuber Cd-accumulator presenting greater shoot Cd concentration), driven by growth differences associated with biomass production.

With respect to Zn, the two cultivars did not show significant differences in total Zn uptake. However, the two cultivars differed with respect to internal Zn partitioning, mainly between the stems and the tubers. The differences in internal distribution were mainly regulated by differences in stem dry weight accumulation. Another interesting observation was that, despite the similar chemical and physical properties of Cd and Zn, partitioning between shoot and tuber was not completely shared. A lower proportion of the total Cd content was translocated to the tubers, compared to Zn. This study identified factors (whole-plant uptake, root-to-shoot translocation, biomass, and shoot-to-tuber partitioning) involved in determining tuber Cd concentration but was unable to identify the most important variable(s) causing differences in tuber Cd concentration between the two cultivars. By assuming all variables do not have an equal contribution to differences in tuber Cd concentration, the research described in the next chapter will evaluate the contribution of individual variables to tuber Cd concentration and identify the most important variables.

Chapter 4: The roles of shoot and root on growth and transition element uptake and distribution in potato

Mengist, M.F., Milbourne, D., Alves, S., McLaughlin, M.J., Jones, P.W. and Griffin, D., 2018. Roles of roots and shoots in cadmium uptake and distribution in tubers of potato (*Solanum tuberosum* L). Plant and Soil (Accepted)

4.1. Introduction

As the majority of Cd and Zn is taken up by the basal roots, transported to the shoot via the xylem and redistributed to the tuber via the phloem (Kratzke & Palta, 1985, 1986; Reid *et al.*, 2003), several physiological processes including root uptake, root-to-shoot translocation, shoot retention and partitioning between shoot and tuber can contribute to the differences in tuber Cd and Zn concentrations.

Studies on soybean (Sugiyama *et al.*, 2007), *Solanum* spp (Arao *et al.*, 2008; Xu *et al.*, 2012a) and the Cd and Zn hyperaccumulator *Noccaea caerulescens* (Sterckeman *et al.*, 2015) found that root-to-shoot translocation is an important factor in determining differences in shoot Cd accumulation. Furthermore, these studies reported the importance of the root in driving Cd uptake. On the other hand, several studies (Clarke *et al.*, 1997; He *et al.*, 2006; Kubo *et al.*, 2008; Yan *et al.*, 2013; Perrier *et al.*, 2016) emphasized the importance of Cd partitioning to the above-ground plant parts. According to these studies, Cd concentrations in the shoot and grain are positively associated and influenced by genotypic variations in shoot biomass, mainly through dilution as a result of dry matter accumulation. Therefore, difference in Cd concentration in the edible portions can be explained by genotypic differences in total Cd uptake, root-to-shoot translocation and above-ground partitioning mediated by shoot biomass (Sugiyama *et al.*, 2007; Arao *et al.*, 2008; Xu *et al.*, 2012a; Sterckeman *et al.*, 2015).

As with the effects on Cd, grain and fruit Zn concentrations are influenced by many factors including root-to-shoot translocation (Hart *et al.*, 1998; Chatzistathis *et al.*, 2009; Wu *et al.*, 2010; Impa *et al.*, 2013), above ground partitioning (Jiang *et al.*, 2008; Yoneyama *et al.*, 2010; Yamaguchi *et al.*, 2012; Yamaji & Ma, 2014) and shoot biomass (AbuHammad *et al.*, 2016; Hussain *et al.*, 2016).

From a mechanistic point view, many factors can influence Cd and Zn concentrations in the edible portions of the plant (Sugiyama *et al.*, 2007; Arao *et al.*, 2008; Xu *et al.*, 2012a; Sterckeman *et al.*, 2015). However, it is presumed that these factors do not make equal contributions to the variation in elements concentrations in the sink. Some factors are more important than others. From a practical plant breeding point of view, it is important to identify the most important and rate-limiting factor(s). In Chapter 3,

the physiological basis of differences in tuber Cd and Zn concentrations was investigated. However, that study (Chapter 3) was limited to identifying the most important factors for differences in tuber Cd and Zn concentrations because of the confounding effects of differences in total Cd uptake, root-to-shoot translocation, shoot Cd and Zn concentrations and biomass, particularly in the foliage.

The first hypothesis was that differences in Cd uptake could be due to inter-cultivar differences in root Cd uptake, which could have a significant contribution to total Cd content. This hypothesis was tested in Chapter 4 using reciprocal grafting between Cara and Lady Rosetta. Reciprocal grafting has also been applied to determine the role of root and shoot for Cd uptake and distribution in different crops including soybean (Sugiyama *et al.*, 2007), spinach (Xin *et al.*, 2013), sweet potato (Xin *et al.*, 2017) and *Solanum nigrum* and *Solanum torvum* (Xu *et al.*, 2012a) and *Solanum melongena* and *Solanum torvum* (Arao *et al.*, 2008). In potato, reciprocal grafting has been used to determine the respective roles of root and shoot on growth and yield (Trudgill & Thompson, 1987), water stress (Jefferies, 1993) and salt tolerance (Shaterian *et al.*, 2005).

Using reciprocal grafting, it is possible to dissect the respective contributions of roots and shoots on total Cd uptake and biomass-driven Cd and Zn distribution between organs of potato cultivars. It was also hypothesized that F₁ genotypes, from a cross between Cara and Lady Rosetta, would vary with respect to shoot Cd and Zn concentrations, and that this variation could be linked to shoot biomass. Furthermore, the variation in shoot Cd and Zn concentrations could influence tuber Cd and Zn concentrations. Therefore, the main objectives of this study were to identify the contribution of differences in total Cd uptake between the cultivars to the differences in tuber Cd concentration, and to determine whether or not differences in shoot biomass accumulation affected tuber Cd and Zn concentration. To achieve these two objectives, the two cultivars and their grafted combinations, and sixteen F₁ genotypes, selected on the basis of maturity class (Chapter 5), from a cross between Cara and Lady Rosetta, were examined for biomass and Cd and Zn concentrations in the various organs of potato.

4.2. Materials and Methods

4.2.1. Grafting experiments

Certified virus-free similar-sized tubers of Cara and Lady Rosetta were sprouted under constant diffused light conditions and planted in 1 L pots filled with peat (Bord Na Mona Horticulture Limited, Ireland) under controlled greenhouse conditions with 16 h light at 21⁰C. Reciprocal grafting between single-stem plants of Cara and Lady Rosetta was carried out when plants reached ca. 15 cm high. The stems were cut ca. 5 cm high from the root-to-stem junction, and then cleft grafting was applied (Jefferies, 1993). The grafted zone was fixed with autoclave tape. The plants were covered with polythene bags and kept under a low light intensity and high relative humidity conditions for 12 days. Non-grafted Cara and Lady Rosetta, and their grafted products (Cara/LR, LR/Cara, Cara/Cara and LR/LR; designated scion/rootstock and LR = Lady Rosetta) were used. Ungrafted plants were planted one week later than the grafted treatments to synchronize the growth status of the ungrafted and grafted plants.

Two grafting experiments were carried out for this study. For the first grafting experiment, successfully grafted and non-grafted plants were transplanted to 10 L pots filled with soil containing 1 mg Cd kg⁻¹ soil (Table 3.1). The second experiment was also carried out under the same conditions except that the soil was amended weekly with 0.8 mg kg⁻¹ Cd as CdCl₂ to attain the final soil Cd concentration of 5 mg kg⁻¹ between 35 to 63 days after transplanting. For both grafting experiments, the transplanted plants were grown under glasshouse conditions with 16 h light at 21⁰C. The soil was supplemented by the addition of 50 g pot⁻¹ NPK(S) (7-6-17(13)) fertilizer, and three biological replicates were used.

4.2.2. Evaluation of genotypes for dry weight, Cd and Zn concentrations

Sixteen F₁ genotypes, from a cross between Lady Rosetta and Cara (Chapter 5) were used for this experiment (Table 4.1). The genotypes were selected from a large mapping population to represent ranges of tuber Cd concentration within each of four maturity classes. Tubers from these F₁ seedlings were raised in peat (Bord Na Mona Horticulture Limited, Ireland) and single-stem uniform seedlings ca. 10 cm high were transplanted to 10 L pots filled with soil naturally containing 1 mg Cd kg⁻¹ soil (Table 3.1) under greenhouse condition. The soil was air dried, crushed, sieved (through a

1.1cm sieve) and homogenised by hand. The soil was supplemented by 50g pot⁻¹ of NPK(S) (7-6-17(13)). The experiment was laid out in a randomized complete block design (RCBD) with three biological replicates. Water was supplied by spraying as required.

4.2.3. Plant harvesting and sampling

Both experiments were harvested 120 days after planting. At harvest, the plants were carefully separated from the soil. The tubers were collected, washed, soaked for 10 minutes in an ice-cold 5 mM CaCl₂ solution and rinsed in distilled water to remove exogenous Cd. Roots of the intact plant were washed carefully with water and immersed for 10 min in an ice-cold 5 mM CaCl₂ solution, followed by rinsing in distilled water while still attached to their shoots. Each plant was then separated into different organs, detopped at the base of the stem and then divided into leaf and stem. Fresh weight of the organs (stem, leaf and root) was recorded immediately, and samples were then oven dried at 70°C until a constant weight was attained. The samples were ground using a mixer mill (Retsch™ MM 400 Mixer Mill, Fisher Scientific, Retsch, Hann, Germany) and stored at room temperature until analysis was carried out. For potato tubers, the samples were homogenised using a vegetable mixer. For the experiment with the selected F₁ clones, the three biological replicates of stem and tuber samples were pooled for chemical analysis.

4.2.4. Cd and Zn analysis

Approximately 1 g homogenized tuber or 0.1 g dried leaf, stem or root powder were microwave digested with 1 ml ultrapure water (resistivity 18 MΩcm⁻¹), 1 ml concentrated nitric acid (HNO₃, 69%, Aristar grade, VWR) and 0.5 ml hydrogen peroxide (H₂O₂, 30%, Sigma-Aldrich). The digested sample was diluted with ultrapure water to 50 ml in a volumetric TPP flask, transferred to a 50 ml falcon tube (TPP, Techno Plastic Products, Switzerland) and kept at 4°C prior to chemical analysis. The samples were analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Perkin Elmer ELAN DRC-e, Monza, Italy). To express tuber Cd (or Zn) concentration on a dry weight (DW) basis, 10 g of homogenized tuber samples were oven dried at 70°C until a constant weight was attained. Dry matter content (%) was calculated by the ratio of DW to fresh weight of the corresponding sample and multiplied by 100. Then, tuber Cd (or Zn) concentrations were adjusted to a DW basis

using the appropriate dry matter percentage. The final figures and statistical analyses were performed using data expressed as mg kg⁻¹ DW.

Table 4.1 List of F₁ genotypes used for this study and their maturity class

No	Genotype	Maturity
1	55	8
2	6	8
3	48	8
4	67	8
5	152	6
6	188	4
7	200	3
8	201	3
9	27	5
10	66	6
11	97	2
12	147	2
13	184	2
14	148	1
15	117	1
16	124	1

Measurements followed the methodology described by Bradshaw *et al.* (2008). Maturity was scored using a 1 to 9 scale with 1 = very late maturing and 9 = very early maturing.

4.2.5. Statistical analysis

Analysis of variance was applied to evaluate differences with respect to dry weight (DW), Cd (or Zn) concentration and content of the different organs (tuber, leaf, stem and root). For all experiments, multiple comparisons were determined by the Tukey test. All statistical analyses were carried out using SAS (SAS 9.4).

4.3.Results

4.3.1. Grafting experiments

Success of grafting was dependent on the scion source (Table 4.2). Plants with a Lady Rosetta scion grafted onto a Cara or Lady Rosetta rootstock had difficulty forming a union at the graft junction. At the junction of a Lady Rosetta scion on a rootstock of Cara, plants often retained water and decayed. On the other hand, Cara scions easily

formed unions with rootstocks from Cara or Lady Rosetta. The data suggest that the two cultivars differed in their ability to form unions at the junction (Table 4.2).

Table 4.2 Evaluation of success of grafting between the two cultivars

Grafted plants	No. of grafted plants	Successful grafts	% success
Cara/Cara	21	12	57
LR/LR	26	11	42
Cara/LR	30	22	73
LR/Cara	56	10	18

LR, Lady Rosetta, Plants are self-grafted Cara (Cara/Cara) and self-grafted Lady Rosetta (LR/LR), and plants with a Cara scion with a Lady Rosetta rootstock (Cara/LR) and a Lady Rosetta scion with a Cara rootstock (LR/Cara). Data represent the number of surviving plants as a percentage of the total number of grafted seedlings of those specific combinations.

4.3.2. Dry weight production and distribution

Canopy height was assessed between 25 and 120 days after planting (Figure 4.1). Grafted plants with Cara scions had greater canopy heights than did those with Lady Rosetta scions, reflecting the importance of the scion in controlling canopy growth. However, the rootstock did not show any influence on canopy growth (Figure 4.1). Regarding biomass production, self-grafted and non-grafted plants of the same genotype did not show significant differences for the measured traits (Figures 4.2A, 4.2C, 4.3A, 4.3C). Generally, grafting did not significantly affect DW accumulation.

At the low soil Cd concentration (1 mg kg^{-1}), there were significant ($P < 0.01$) differences for stem, root and leaf DW between the graft combinations. Plants with a Cara scion grafted onto a Lady Rosetta rootstock did not show any differences from self-grafted Cara or non-grafted Cara with respect to shoot and root DW. In the same fashion, plants with a Lady Rosetta scion grafted onto a Cara or a Lady Rosetta rootstock had a level of biomass production in the shoot and root comparable with that of self-grafted Lady Rosetta or non-grafted Lady Rosetta. Basically, plants with the same scion had similar level of biomass production and canopy height regardless of the cultivar of the rootstock (Figure 4.2A), suggesting that the scion of the cultivar largely determined shoot (and, to some extent, root) DW.

Another interesting observation was that plants differed with respect to DW partitioning between organs (Figure 4.2). Plants with Cara scions allocated more DW to the stems than the leaves whilst plants with Lady Rosetta scions allocated more DW to the leaves than the stems (Figure 4.2A). Overall, DW was greater in plants with a Cara scion than in plants with a Lady Rosetta scion (Figure 4.2C). No significant differences in tuber DW were observed for any of the plants in the experiment, regardless of grafting status (Figure 4.2C; 4.3C).

4.3.3. Cd concentration and content in the grafted plants

Like the effects on DW production, Cd concentration and Cd content were similar between non-grafted and self-grafted plants with respect to all organs (Figures 4.2B, 4.2D; 4.3B, 4.3D) indicating that the act of grafting did not significantly affect Cd concentration or content.

At the 1 mg Cd kg⁻¹ soil level, the grafted plants showed significant ($P < 0.05$) differences in stem, leaf, root and tuber Cd concentrations (Figures 4.2B, 4.4A). Plants with a Cara scion grafted onto a Lady Rosetta rootstock exhibited a greater Cd concentration in the leaves, stems and tubers compared with non-grafted or self-grafted Cara. Conversely, plants with a Lady Rosetta scion grafted onto a Cara rootstock had significantly less leaf and stem Cd concentrations compared to self-grafted Lady Rosetta (Figures 4.2B, 4.4A).

With respect to organ Cd content, the grafted plants showed significant ($P < 0.05$) differences with respect to the stem, leaf, root and tuber in plants grown at the 1 mg Cd kg⁻¹ soil level. Interestingly, plants with a Cara scion grafted onto a Lady Rosetta rootstock had greater Cd content in the stem and leaf than self-grafted Cara or non-grafted Cara. On the other hand, plants with a Lady Rosetta scion grafted onto a Cara rootstock had less Cd accumulation than self-grafted Lady Rosetta or non-grafted Lady Rosetta (Figure 4.2D).

Examination of Cd uptake differences between grafted plants at the 1 mg Cd kg⁻¹ soil level (Figure 4.4B) showed that plants with a Cara scion grafted onto a Lady Rosetta rootstock increased total Cd uptake by 31%, compared to self-grafted Cara. Contrastingly, plants with a Lady Rosetta scion grafted onto a Cara rootstock reduced total Cd uptake by 37% compared to self-grafted Lady Rosetta (Figure 4.4B). Despite

the significantly greater plant Cd exhibited by a Cara scion with a Lady Rosetta rootstock compared to that of a self-grafted Cara, tuber Cd concentration was increased by only 18%, relative to self-grafted Cara (Figures 4.4A, 4.4B). On the other hand, plants with a Lady Rosetta scion grafted onto a Cara rootstock increased tuber Cd concentration by 56% compared with self-grafted Cara (Figures 4.4A, 4.4B).

At the high soil Cd (5 mg Cd kg⁻¹ soil level), the trends of DW accumulation, Cd concentration and Cd content between treatments resembled those observed at the lower Cd (Figures 4.3, 4.5). Specific to total plant Cd uptake, plants with a Cara scion grafted onto a Lady Rosetta rootstock had 39% greater total Cd uptake than self-grafted Cara. Conversely, plants with a Lady Rosetta scion grafted onto a Cara rootstock had a total Cd uptake 19% less than self-grafted Lady Rosetta (Figure 4.5B). Total Cd uptake differences were less important, with only 21% larger tuber Cd concentration being recorded when Cara was grafted onto Lady Rosetta rootstock, compared to self-grafted Cara. On the other hand, Lady Rosetta grafted onto Cara had two-fold more tuber Cd concentration than self-grafted Cara or ungrafted Cara (Figure 4.5A). These results suggest that differences in tuber Cd concentration are largely determined by the scion of the genotype and to some extent by genotypic difference in total Cd uptake. Another interesting observations at high soil Cd was that Cd concentration in the root was much greater than that in other organs. Moreover, plants with a Lady Rosetta scion had significantly ($P < 0.01$) greater root Cd concentration than did plants with a Cara scion (Figure 4.3B), indicating that the Lady Rosetta scion is important for regulating root-to-shoot Cd concentration and/or translocating Cd from shoot to root.

Overall, the rootstock proved to be an important organ in driving Cd uptake but was less important for determining tuber Cd concentration. On the other hand, the scion was an important factor for the determination of growth and Cd concentration between organs. However, it is not fully understood why the scion of the cultivar was important for regulation of differences Cd concentration between organs, cultivar-specific preferential distribution or simple biomass driven dilution effects. The mode-of-action was investigated in the next part of this study, using 16 F₁ genotypes derived from these two cultivars.

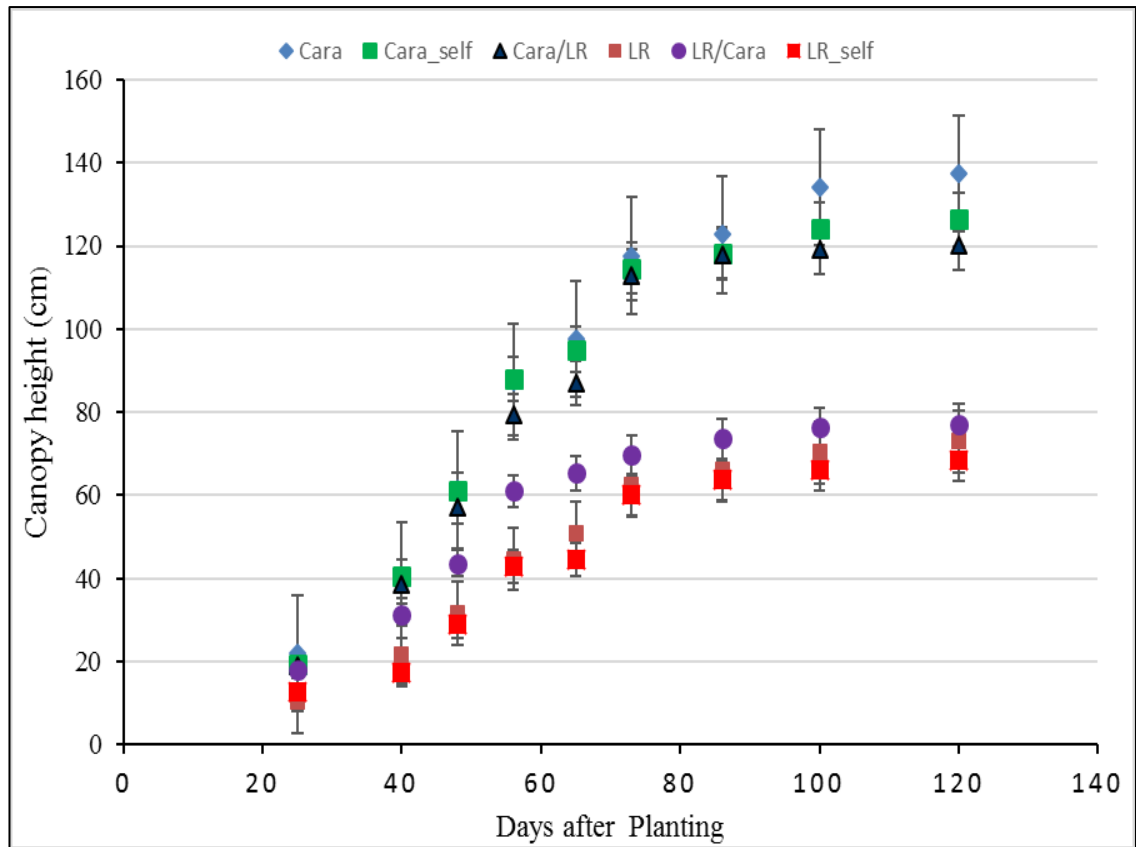


Figure 4.1 Plant height change in reciprocal grafts over time. Error bars represent the standard error of the mean (n=3). LR= Lady Rosetta.

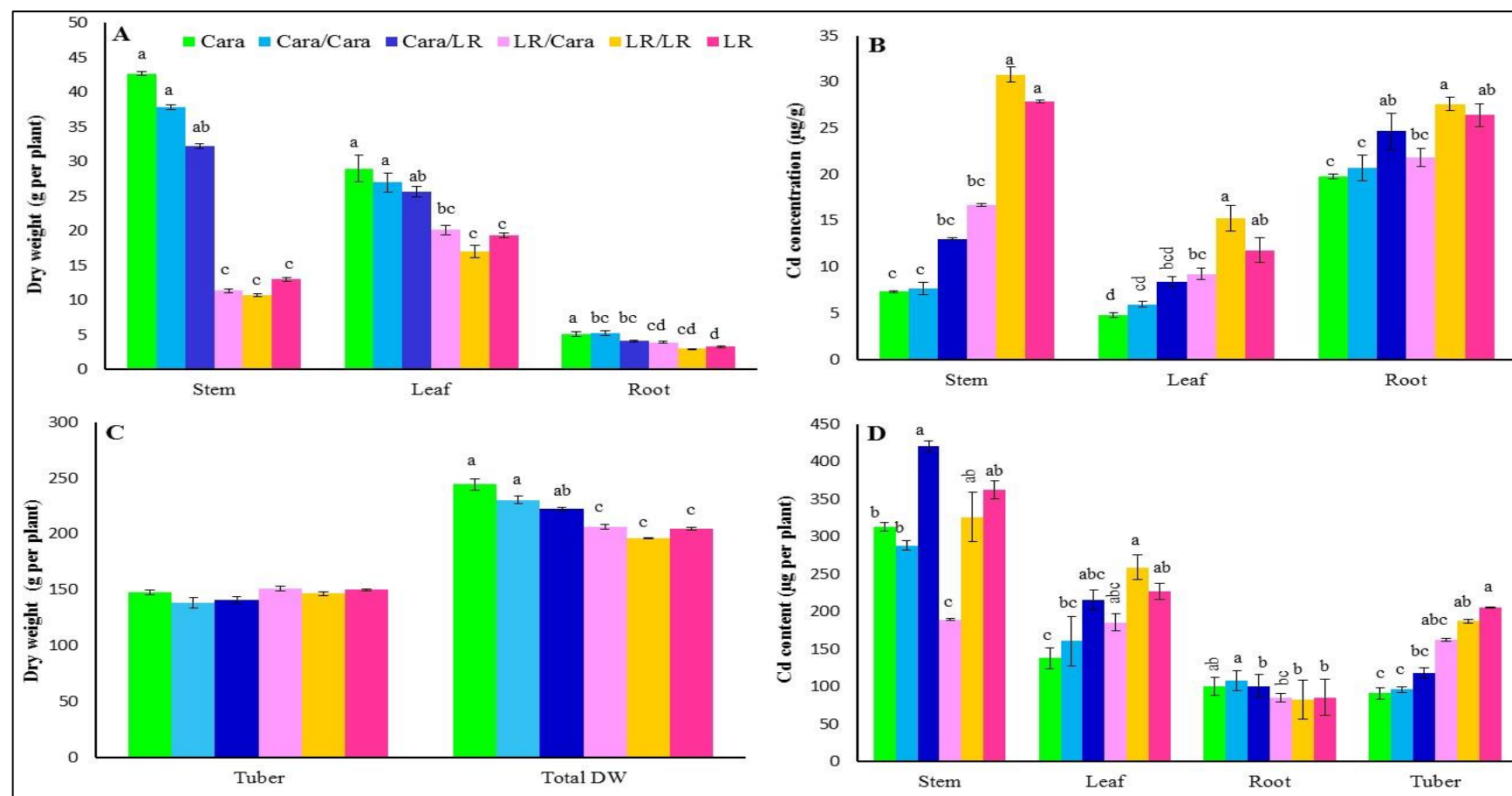


Figure 4.2 DW accumulation (A) and Cd concentrations (on DW basis) (B) in the leaf, stem and root; tuber and whole plant DW (C) and Cd content in organs (D) of the treatments at the 1 mg kg⁻¹ soil Cd. Mean values with a common letter are not significantly different. Error bars represent the standard error of the mean (n=3). Plants are self-grafted Cara (Cara/Cara) and self-grafted Lady Rosetta (LR/LR), and plants with a Cara scion with a Lady Rosetta rootstock (Cara/LR) and a Lady Rosetta scion with a Cara rootstock (LR/Cara). Mean values with common letters are not significantly different

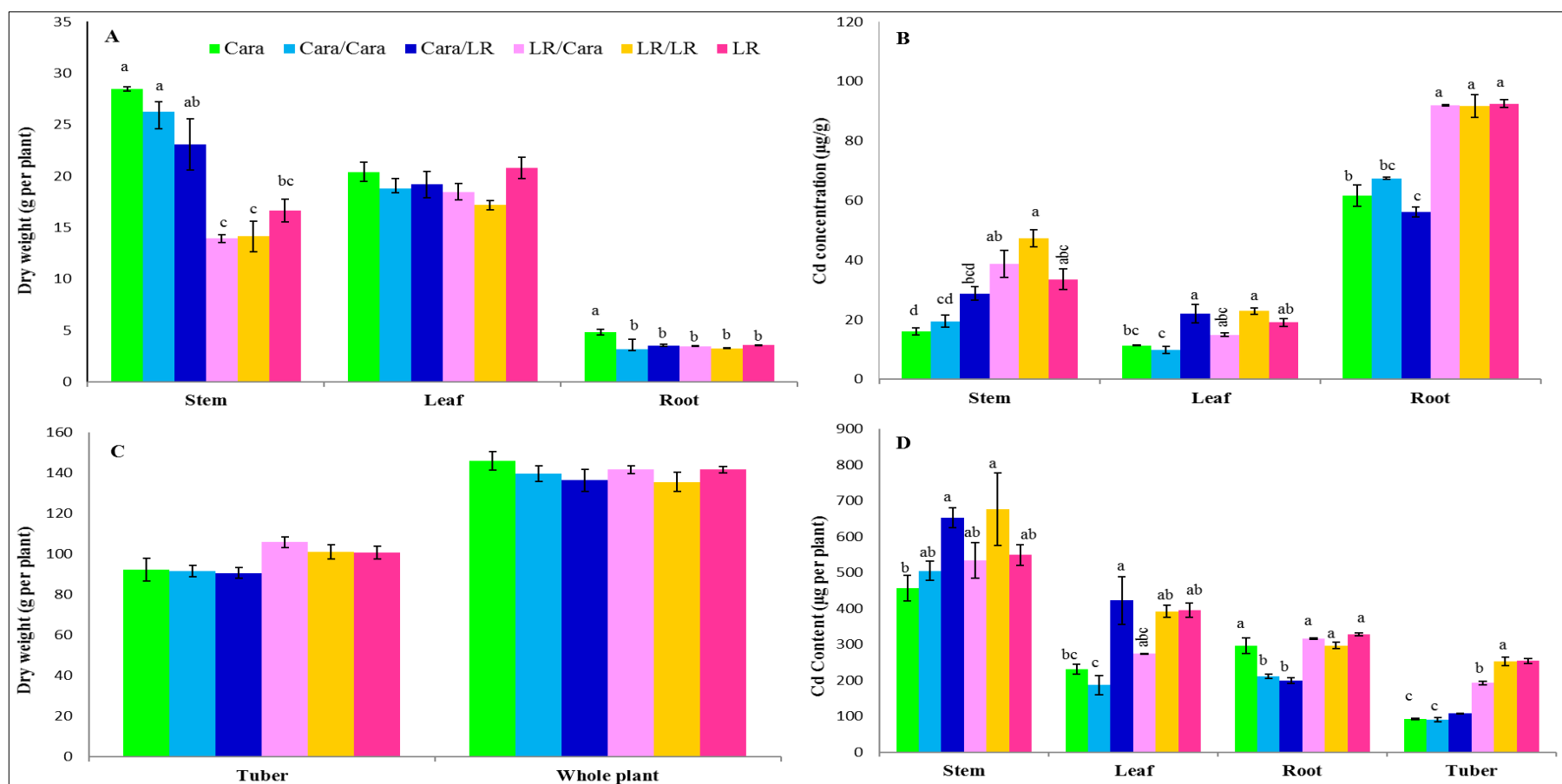


Figure 4.3 DW accumulation (A) and Cd concentration (DW basis) (B) in leaf, stem and root, tuber and whole plant DW (C) and Cd content in organs (D) of the treatments at 5 mg kg⁻¹ soil Cd. Mean values with common letter are not significantly different. Error bars represent the standard error of the mean (n=3). LR, Lady Rosetta. Plants are self-grafted Cara (Cara/Cara) and self-grafted Lady Rosetta (LR/LR), and plants with a Cara scion with a Lady Rosetta rootstock (Cara/LR) and a Lady Rosetta scion with a Cara rootstock (LR/Cara). Mean values with common letters are not significantly different

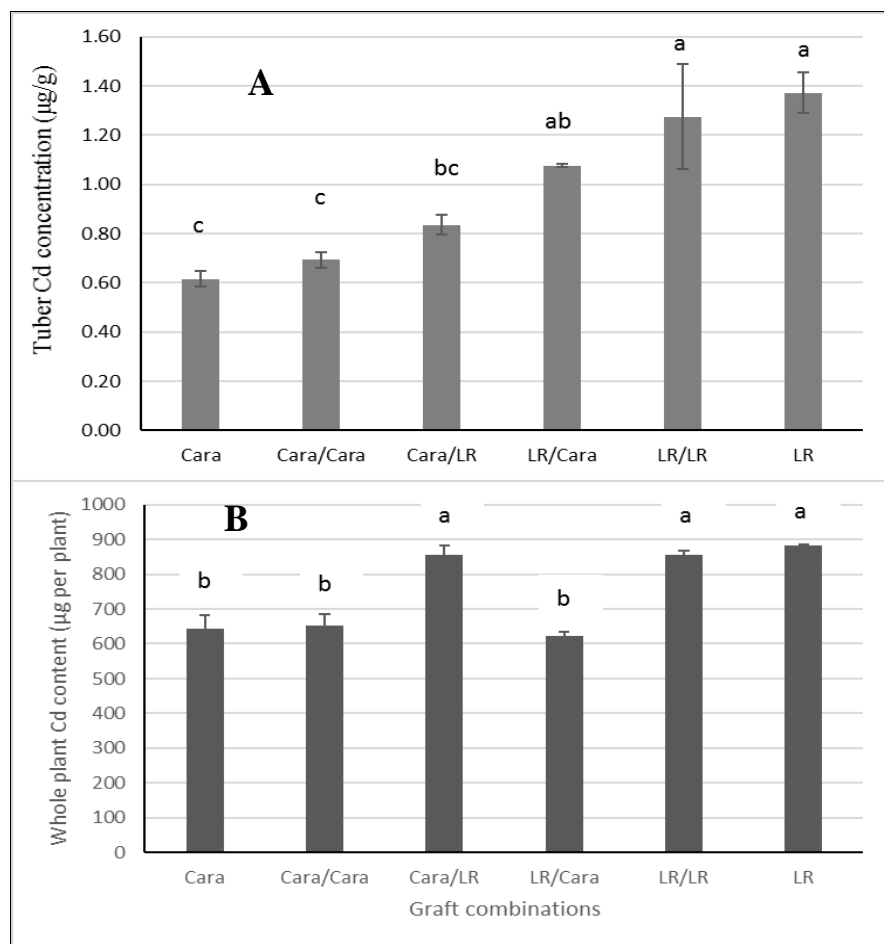


Figure 4.4 Tuber Cd concentration (DW basis) (A) and whole plant Cd accumulation (B) at the 1 mg kg⁻¹ soil Cd level. Mean values with common letters are not significantly different. Error bars represent the standard error of the mean (n=3). LR, Lady Rosetta.

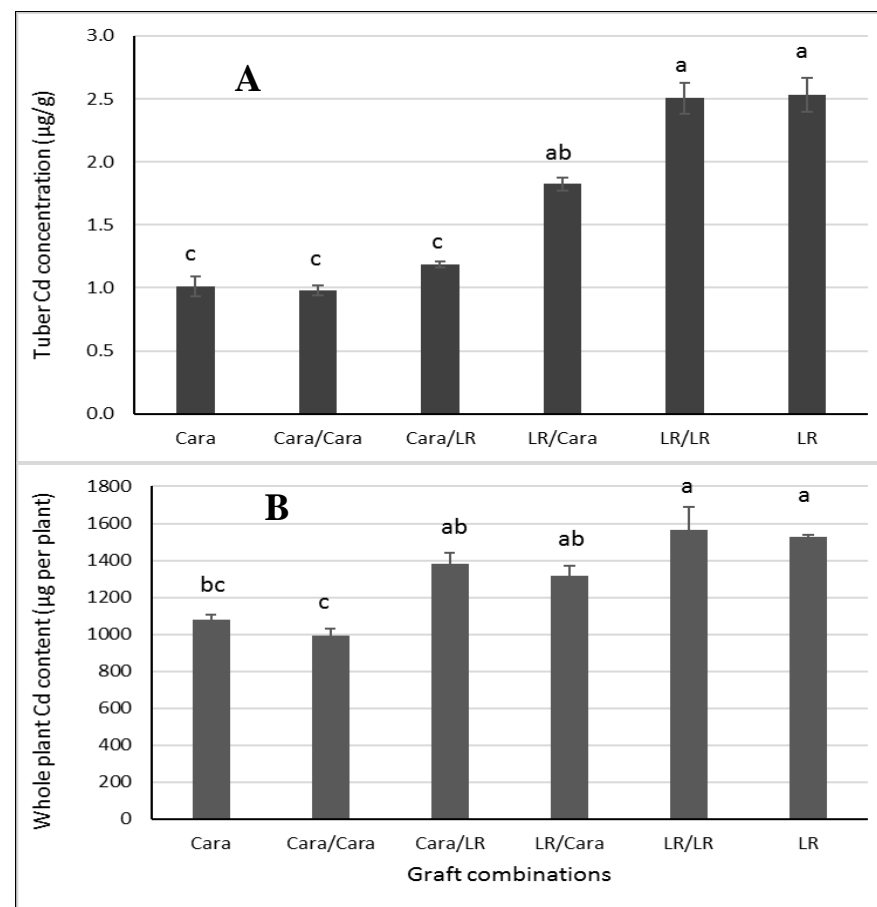


Figure 4.5 Tuber Cd concentration (DW basis) (A) and whole plant Cd accumulation (B) of plants grown at the 5 mg kg⁻¹ soil Cd level. Mean values with common letter are not significantly different. Error bars represent the standard error of the mean (n=3). LR, Lady Rosetta.

4.3.4. Zn concentration and content between the grafted plants

The results showed that there was no significant difference between self-grafted and non-grafted plants of the same cultivars for organ Zn concentration, organ Zn content or whole-plant Zn accumulation at low soil Cd (Figures 4.6, 4.7), suggesting grafting did not affect Zn distribution or total Zn uptake. For plants grown at the high soil Cd, there was significant ($P < 0.05$) difference between self-grafted and non-grafted plants of the same cultivars for root Zn concentration (Figure 4.8).

At the low soil Cd, the grafted combinations showed significant ($P < 0.05$) differences in terms of stem and tuber Zn concentrations (Figure 4.6). According to these results, non-grafted Lady Rosetta or self-grafted Lady Rosetta had greater Zn concentrations in the stem or tuber than did self-grafted Cara or ungrafted Cara. Surprisingly, reciprocal grafted combinations did not differ significantly from Cara or Lady Rosetta with respect to tuber Zn concentration, suggesting that both scion and rootstock contributes to the differences in tuber Zn concentration between the cultivars. Self-grafted Lady Rosetta accumulated a greater stem Zn concentration than did plants with Cara scion grafted onto Lady Rosetta rootstock. On the other hand, grafted combinations did not show significant differences in terms of leaf or root Zn concentration (Figure 4.6).

With respect to Zn content, grafted combinations were significantly ($P < 0.05$) different for all measured traits (Figure 4.7). Regardless of the source of the rootstock, plants with the Lady Rosetta scion allocated more Zn to the tuber than did plants with the Cara scion. Conversely, plants with the Cara scion retained much more Zn in the stem than did plants with the Lady Rosetta scion. These results established that the two cultivars differed in terms of Zn partitioning between the stem and the tuber. On the other hand, root-to-shoot translocation of Zn was effective, as no more than 7% of the total Zn was allocated to the root (Figure 4.7). No specific patterns were observed between grafted plants in leaf and root Zn content.

Plants also showed variation in whole-plant Zn uptake. Plants with a Cara scion had slightly more total Zn uptake than did plants with a Lady Rosetta scion. However, plants with Lady Rosetta scion had greater Zn content than Cara scion plants (Figure

4.7). In general, difference in tuber Zn concentration was mainly associated with inter-cultivar differences in Zn partitioning between the shoot and the tuber. Overall, the results of the grafting experiment were consistent with the spatio-temporal accumulation of Zn, that stem played an important role in determining differences in tuber Zn accumulation between potato cultivars.

With respect to plants grown at the high soil Cd, there were significant ($P<0.05$) differences in Zn concentrations in the stems, leaves, roots and tubers between the different grafted combinations. Plants with a Lady Rosetta scion had a greater Zn concentrations in the stems and tubers than did plants with a Cara scion. Leaf Zn concentration was slightly larger in plants with a Lady Rosetta than in ones with a Cara scion. However, the roots did not show a specific pattern with regard to Zn concentration, regardless of the scion or rootstock source (Figure 4.8). With respect to Zn content, plants with a Cara scion had a more Zn concentration in the stem than plants with a Lady Rosetta scion. On the other hand, plants with a Lady Rosetta scion allocated a greater proportion of total Zn to the tubers than did plants with a Cara scion, regardless of the source of the rootstock (Figure 4.8). In general, differences in tuber Zn concentration was mainly accounted for by inter-cultivar differences in Zn partitioning between shoot and tuber. The data also suggested that root-to-shoot translocation of Zn was effective as not more than 7% of the total Zn was allocated to the root, a similar figure to that reported under the low soil Cd.

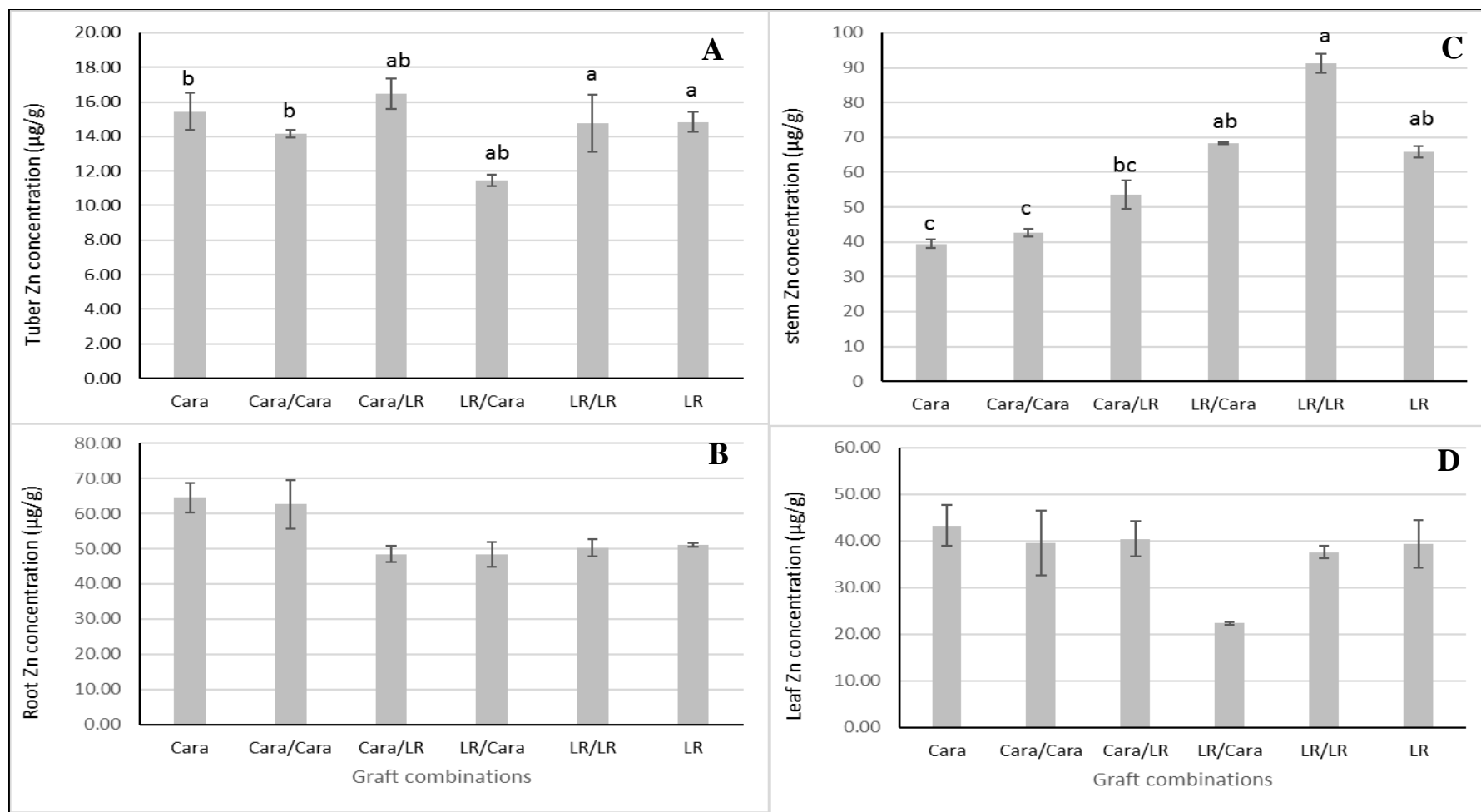


Figure 4.6 Tuber Zn concentrations (DW basis) (A), root Zn concentration (B), stem Zn concentration (C) and leaf Zn concentration (D). Mean values with common letters are not significantly different. Error bars represent the standard error of the mean (n=3). LR, Lady Rosetta, Plants are self-grafted Cara (Cara/Cara) and self-grafted Lady Rosetta (LR/LR), and plants with a Cara scion with a Lady Rosetta rootstock (Cara/LR) and a Lady Rosetta scion with a Cara rootstock (LR/Cara).

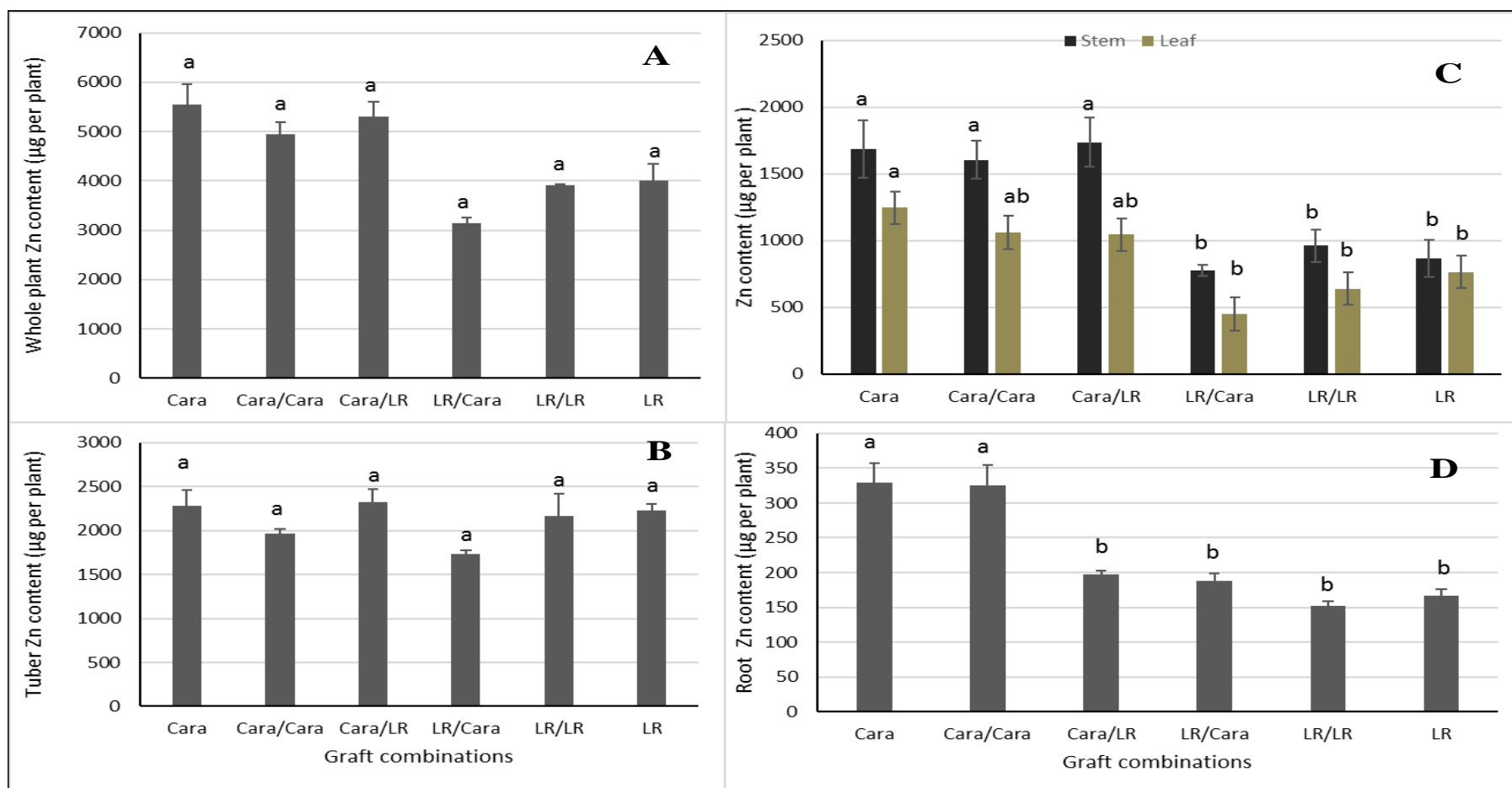


Figure 4.7 Zinc content (on a DW basis) in the whole plant (A), tuber (B), stem and leaf (C) and root (D). Mean values within a graph with common letters are not significantly different. Error bars represent the standard error of the mean (n=3).LR, Lady Rosetta, Plants are self-grafted Cara (Cara/Cara) and self-grafted Lady Rosetta (LR/LR), and plants with a Cara scion with a Lady Rosetta rootstock (Cara/LR) and a Lady Rosetta scion with a Cara rootstock (LR/Cara).

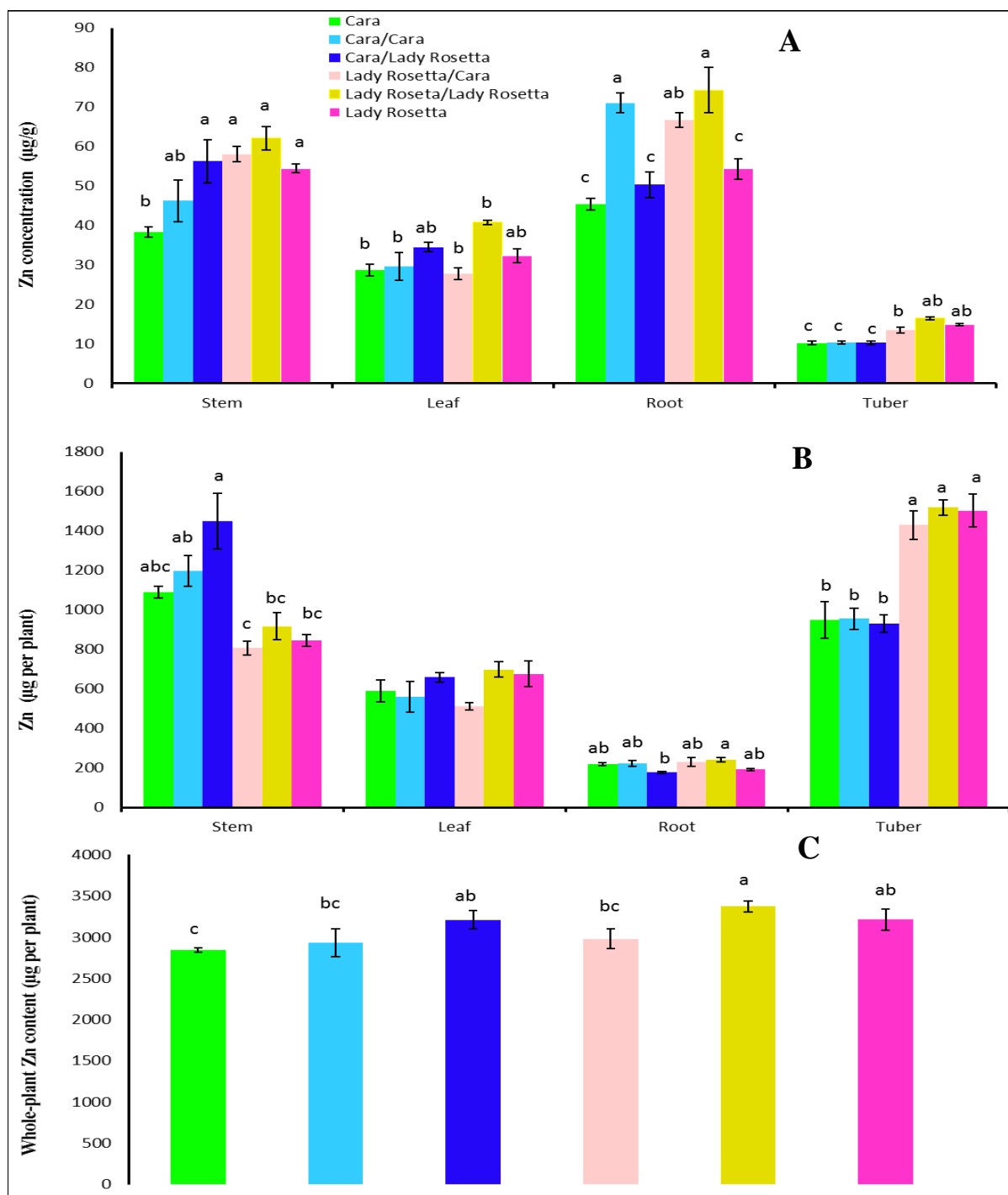


Figure 4.8 Zn concentration (A) and Zn content (B) between organs of the treatments and whole plant Zn content (C) of plants grown at 5 mg kg⁻¹ soil Cd. Mean values with common letter are not significantly different. Error bars represent the standard error of the mean (n=3).

4.3.5. Evaluation of selected F₁ genotypes for DW, Cd and Zn concentrations

Genotypic variation for biomass production

Examination of 16 F₁ genotypes derived from a cross between Cara and Lady Rosetta showed significant ($P<0.01$) differences in root, stem and leaf dry weight accumulations (Figures 4.9A, 4.9B). Late-maturing genotypes generally had greater root and shoot biomass than early-maturing genotypes (Figures 4.9A, 4.9B). Results from this study showed that genotypic difference in leaf biomass was approximately 2.2 fold, which was markedly less than the 4- and 5-fold genotypic variation in root and stem dry weight accumulations, respectively (Figures 4.9A, 4.9B). Among the organs, stem tended to show the greatest variation between genotypes.

Genotypic variation in Cd and Zn concentrations between organs

Significant ($P<0.05$) differences were observed between genotypes in root and leaf Cd concentrations (Figure 4.10). The genotypes showed a 2.28- and 2.87-fold variation for leaf and root Cd concentration, respectively. For stem and tuber, the three biological replications were pooled in order to carry out chemical analysis and hence no statistical analysis was performed. However, the genotypes showed approximately five- and three-fold variations in the stem and tuber Cd concentrations, respectively (Figures 4.10A, 4.11A). The genotypes also showed a 3.54- and 2.60- fold variation in stem and tuber Zn concentrations, respectively (Figures 4.10B, 4.11B). Similar to the variation in Cd concentration, genotypic variation was greater in the stem than in other organs with respect to Zn concentration.

Compared to Cd concentration, Zn concentration exhibited less variation between the genotypes in all measured traits (Figures 4.10B, 4.11B), suggesting that Zn is more highly regulated than Cd, being an essential element for plants. In general, the stem showed greater variation in dry weight accumulation, Cd and Zn concentrations than did the other organs.

Correlation between variables

Variables related to biomass accumulation and Cd and Zn concentrations were analyzed by principal component analysis (PCA). The first two principal components

accounted for approximately 70% of the total variance (Figure 4.12). The results clearly showed that biomass production was strongly associated with maturity, with late-maturing genotypes accumulating more shoot and root biomass than early-maturing genotypes. Conversely, biomass in the stem, leaf and root was inversely correlated with Cd concentration in the stem, leaf and tuber. However, root Cd concentration was less influenced by root or shoot DW.

The trends were somewhat different for tuber Zn concentration. Leaf biomass did not show any association with leaf Zn concentration. Furthermore, leaf and tuber Zn concentrations were negatively associated, implying that the two organs are competing for Zn. Stem biomass negatively affected stem and tuber Zn concentrations. Very strong positive correlations between Cd and Zn concentrations were found in the stem but not in the leaves (Figure 4.12, Table 4.3).

Figure 4.13 presents the Zn/Cd ratio in organs of the 16 selected F₁ genotypes. The data suggest that relatively more Cd was retained in the leaf and less was translocated to the tuber. In the case of Zn, more Zn than Cd was translocated to the tuber, implying that Zn is preferentially transported to the tubers while leaves preferentially retained Cd. The results are consistent with the correlation analysis that Zn is less regulated by the shoot biomass and transpiration.

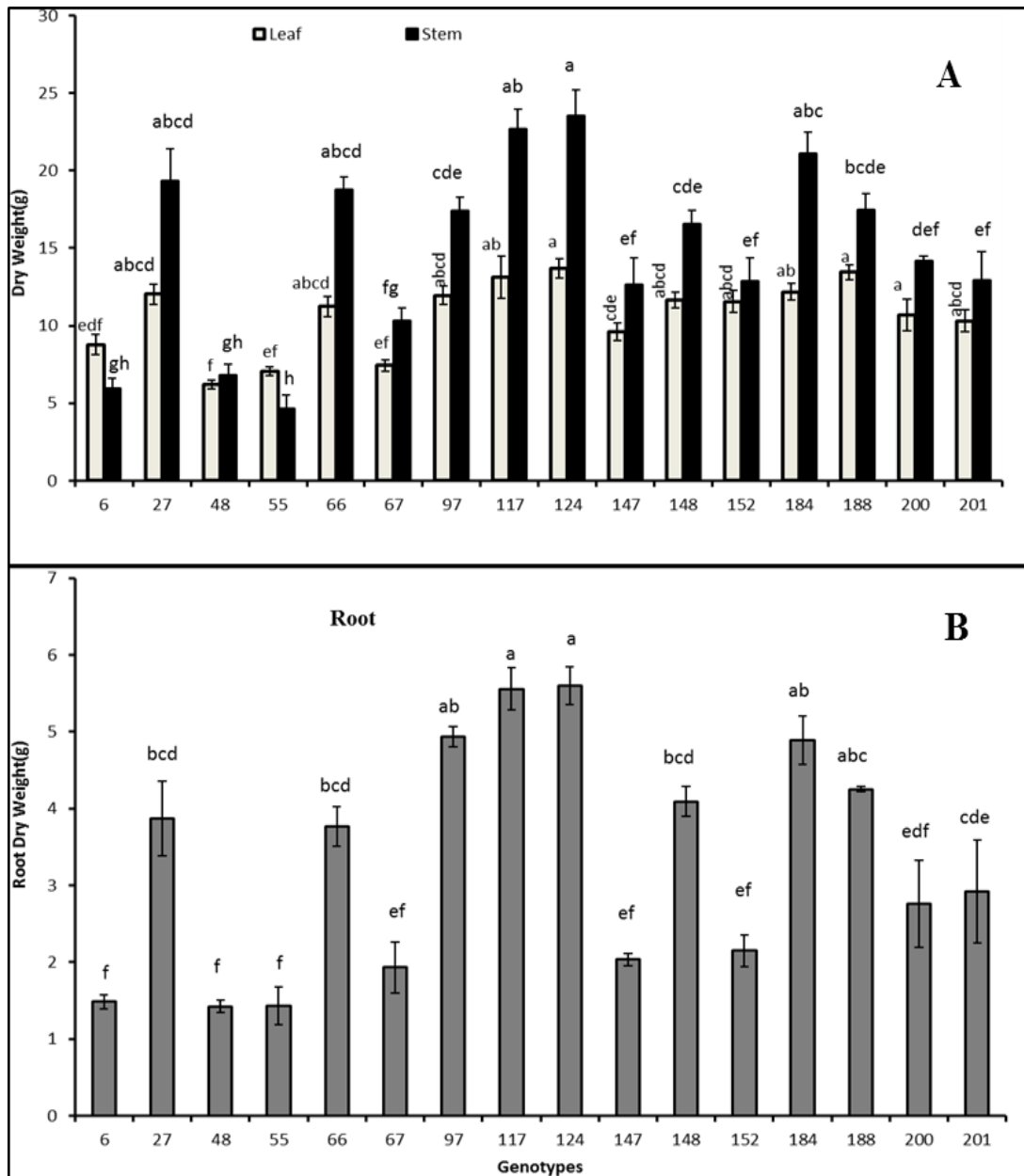


Figure 4.9 DW accumulataton between organs of 16 F₁ genotypes, leaf (A), root (B) and stem (C), measured at 120 DAP. Mean values with common letters are not significantly different as determined by a Tukey test. Error bars represent the standard error of the mean (n=3).

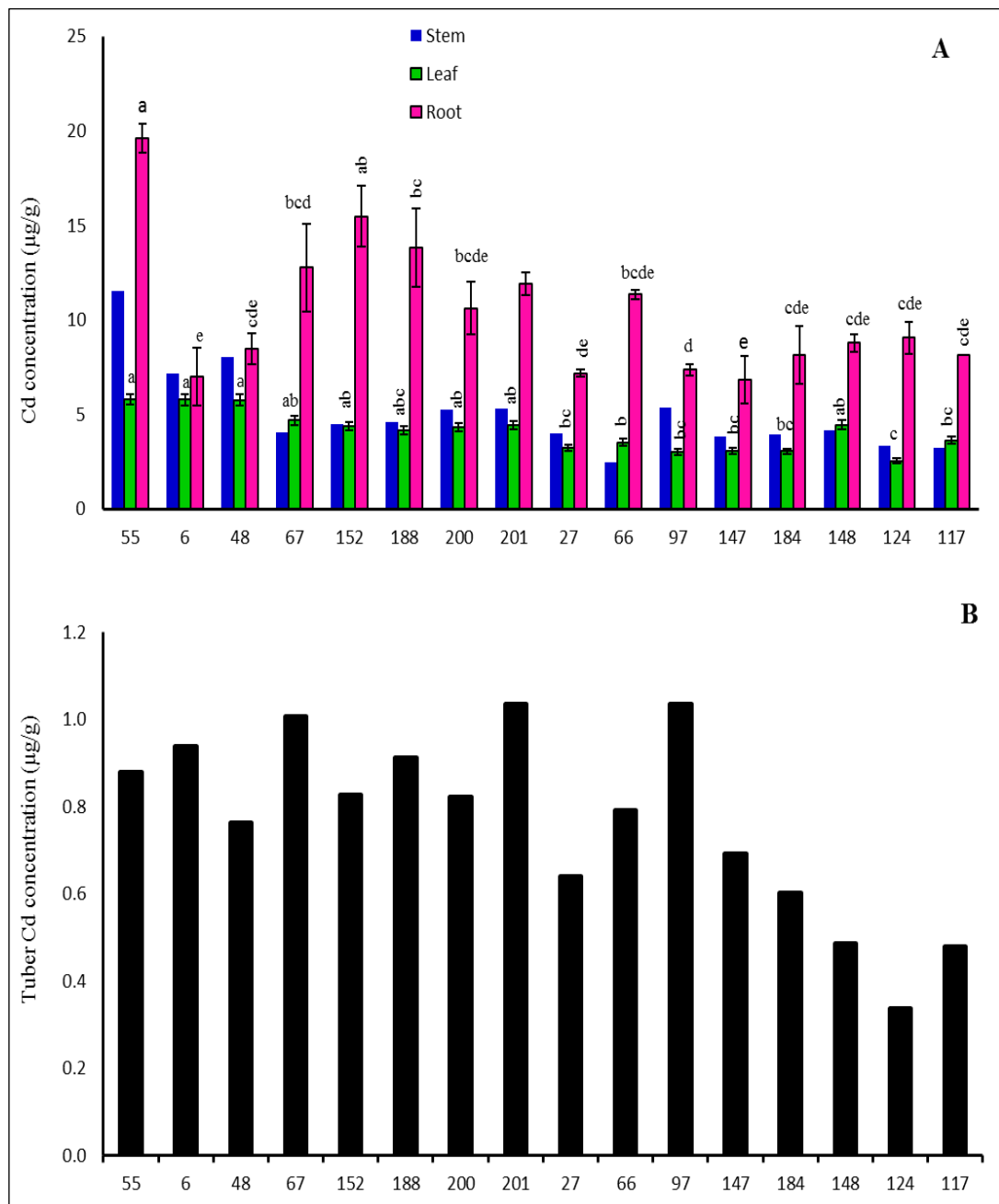


Figure 4.10 Cd concentrations ($\mu\text{g g}^{-1}$ DW) measured at 120 DAP in 16 F_1 genotypes of leaf, stem and root (A) and tuber (B). Mean values with common letter are not significantly different. Error bars represent the standard error of the mean ($n=3$).

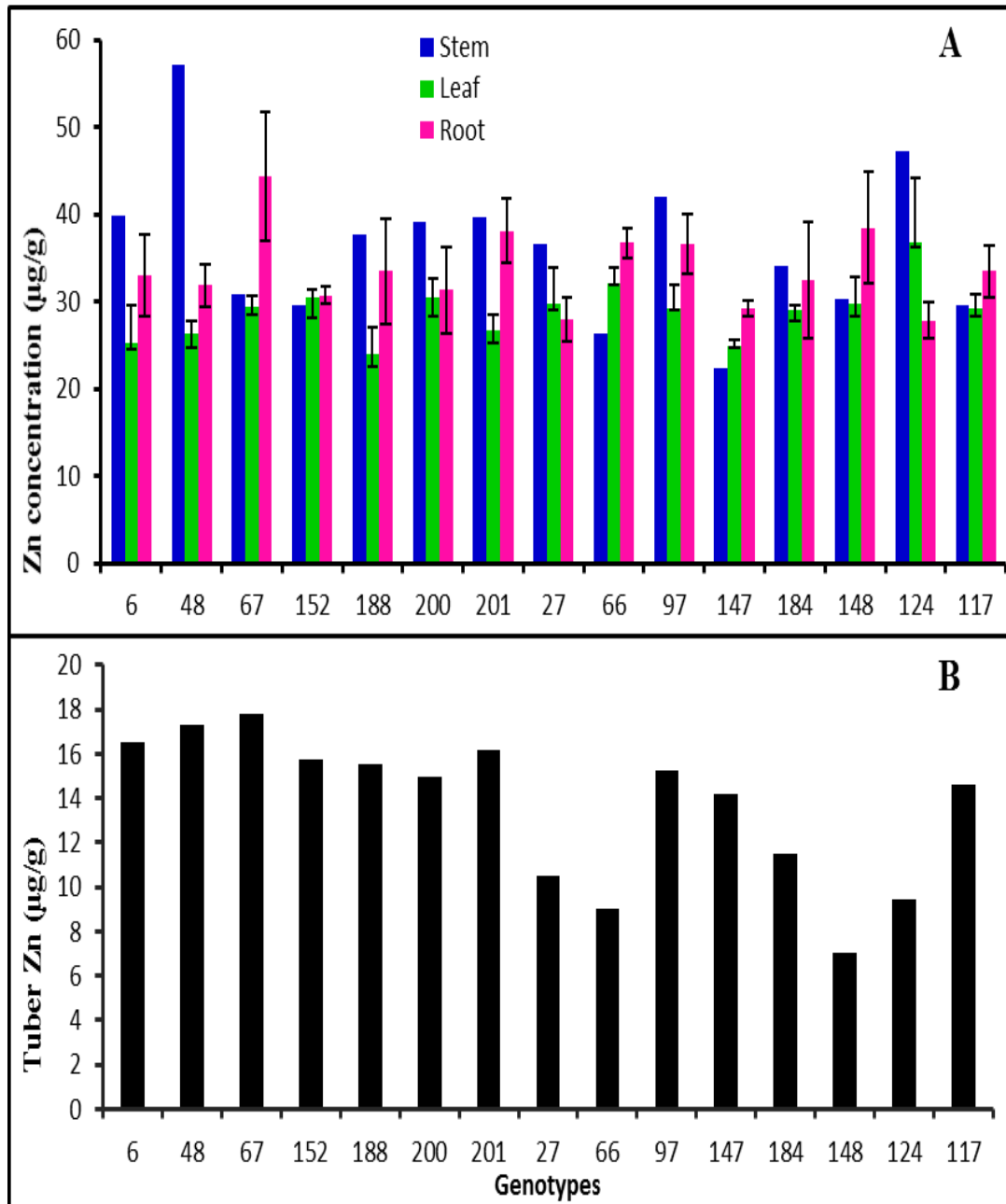


Figure 4.11 Zn concentrations ($\mu\text{g g}^{-1}$) measured at 120 DAP for 16 F_1 genotypes in leaf, stem and root (A) and tuber (B). Error bars represent the standard error of the mean ($n=3$).

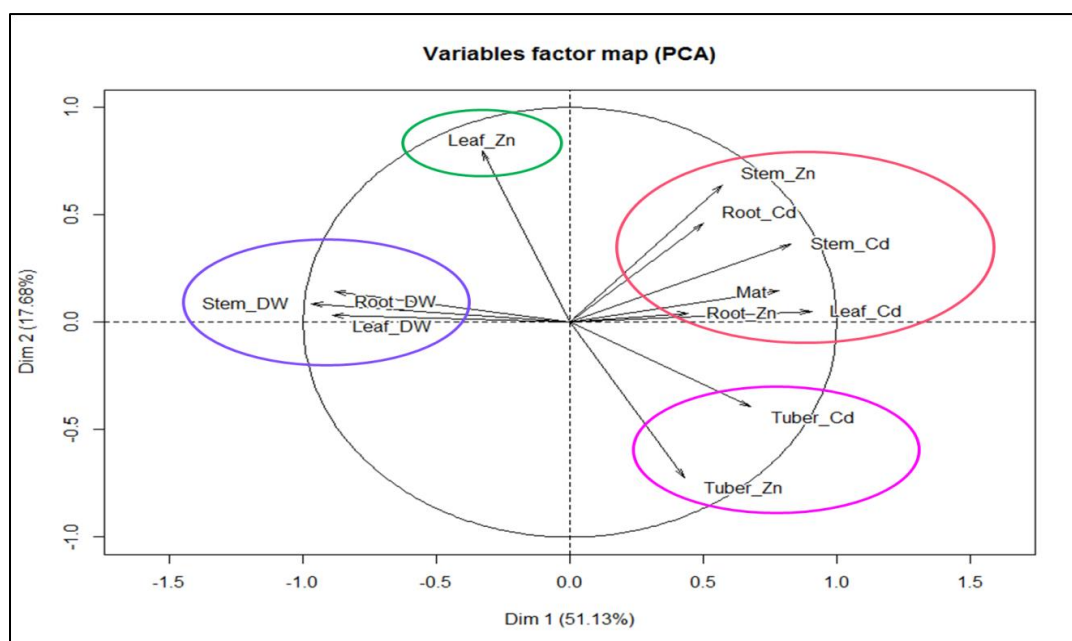


Figure 4.12 Principal component analyses on variables related to DW, and organ Cd and Zn concentrations. The variables are root and shoot biomass (root, stem and leaf DW); Cd and Zn concentration in leaf, stem, root and tuber; maturity (mat-scored 1=very late, 9=very early).

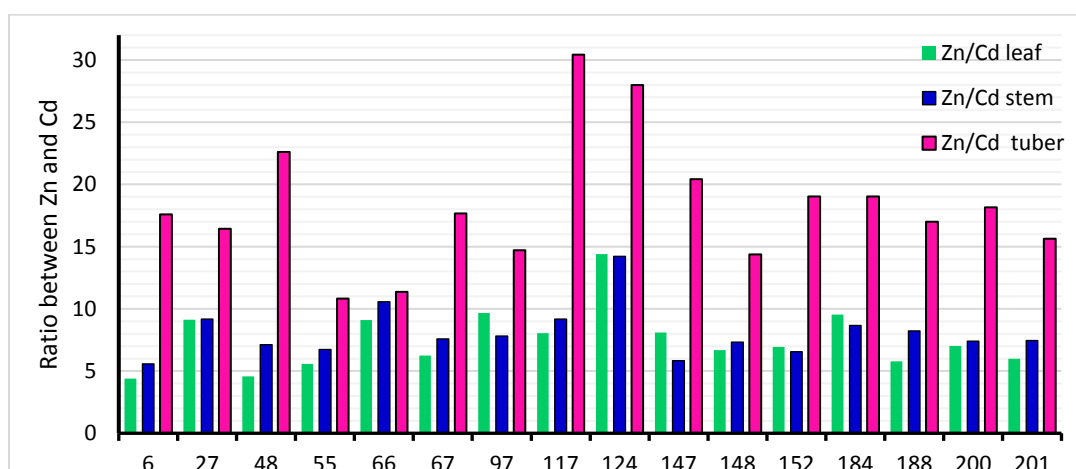


Figure 4.13 The ratio between Cd and Zn concentration in organs of 16 selected F₁ genotypes. It is calculated as $[Zn]/[Cd]$; the larger ratio indicates that more Zn is translocated to or accumulated in that organ compared to Cd and vice versa. Zn/Cd tuber, Zn concentration in tuber divided by Cd concentration in the tuber of that specific genotype; Zn/Cd stem, Zn concentration in the stem divided by stem Cd concentration that specific genotype; Zn/Cd leaf, Zn concentration in leaf divided by leaf Cd concentration of the given genotype.

Table 4.3 Correlation between DW, Cd and Zn concentration in different organs of 16 selected F₁ genotypes

	Root DW	Stem DW	Leaf Cd	Root Cd	Stem Cd	Tuber Cd	Leaf Zn	Root Zn	Stem Zn	Tuber Zn
Leaf DW	0.82**	0.86**	-0.65**	-0.22	-0.64**	-0.44**	0.12	-0.27	-0.42**	-0.31*
Root DW		0.89**	-0.71**	-0.32*	-0.60**	-0.52**	0.18	-0.11	-0.28	-0.41**
Stem DW			-0.78**	-0.35*	-0.77**	-0.58**	0.23	-0.23	-0.46**	-0.41**
Leaf Cd				0.43**	0.70**	0.42**	-0.13	0.27	0.47**	0.34*
Root Cd					0.41**	0.33*	0.07	0.34*	0.41**	0.06
Stem Cd						0.39*	-0.07	0.15	0.88**	0.15
Tuber Cd							-0.29*	0.34*	0.16	0.63**
Leaf Zn								-0.05	0.15	-0.40**
Root Zn									0.08	0.06
Stem Zn										-0.07

**, * significant at $P<0.01$ and $P<0.05$ respectively; DW, Dry weight per plant (g); Cd, Zn concentration ($\mu\text{g g}^{-1}$).

4.4. Discussion

In the current study, by integrating grafting experiments with the analysis of selected F₁ genotypes, derived from a cross between Lady Rosetta and Cara, the most important factors for differences in tuber Cd and Zn concentrations have been identified. According to this study, the rootstock is an important factor for whole-plant Cd uptake (Figures 4.4,4.5). However, shoot and root biomass accumulation, Cd and Zn concentration and partitioning between organs are predominantly controlled by the scion (Figures 4.1,4.4,4.5). Specific to Zn, Zn partitioning between the tubers and the stems is the major cause of differences in tuber Zn concentration (Figures 4.6,4.7).

4.4.1. The scion controls shoot and to some extent root biomass

In this study, it was clearly observed that the scion genotype had a dominant influence on the growth of the shoot and, to some extent, on that of the root. Plants with a Cara scion grafted onto a Lady Rosetta rootstock did not show any biomass accumulation differences in the shoot and root compared to self-grafted Cara or non-grafted Cara. In the same fashion, plants with a Lady Rosetta scion grafted onto a Cara or a Lady Rosetta rootstock had a comparable level of biomass production in the shoot and roots to that in self-grafted Lady Rosetta or non-grafted Lady Rosetta. Simply, plants with the same scion had similar level of biomass production and canopy height, irrespective of the genotype of the rootstock. These results are consistent with previous findings by Trudgill and Thompson (1986) and Jefferies (1993), in that the scion had a greater influence than the rootstock on the partitioning of assimilates and shoot growth of potato cultivars. A recent study showed that the phytohormone abscisic acid (ABA), derived from the shoot, promoted root growth in three plant species, including tomato, a close family of potato (McAdam *et al.*, 2016).

Further analyses of the selected F₁ genotypes, derived from a cross between the same two potato cultivars (Figure 4.12), strengthened the hypothesis that shoot and root biomass are positively correlated traits in potato. Furthermore, the data showed that late-maturing genotypes tended to accumulate greater shoot and root biomass than did early- maturing genotypes. These results are consistent with previous findings (Trudgill & Thompson, 1987; Jefferies, 1993), that differences in maturity class reflect the duration of shoot growth, which is determined by the scion. Results from this study

clearly showed the importance of the genotype of the scion to determine biomass production and growth, which is determined in turn by the genotype of the scion (Figures 4.4, 4.5, 4.4.7, 4.12).

4.4.2. The rootstock drives total Cd uptake but not that of Zn

In a previous study (Chapter 3), Lady Rosetta showed 20 % greater total Cd uptake than did Cara. Consistent with that finding, this current study showed that total Cd uptake was greater in plants with a Lady Rosetta rootstock than in those with a Cara rootstock, regardless of the genotype of the scion (Figures 4.4, 4.5). With regard to grafted plants, a Cara scion grafted onto a Lady Rosetta rootstock had greater total plant Cd uptake than did non-grafted Cara or self-grafted Cara. On the contrary, plants with a Lady Rosetta scion grafted onto a Cara rootstock had less plant Cd accumulation than did non-grafted Lady Rosetta or self-grafted Lady Rosetta (Figures 4.4, 4.5). Therefore, it is the rootstock, not the scion, that determines total Cd uptake. Similar results have been reported in other studies on cucumber (Savvas *et al.*, 2013), the Cd and Zn hyperaccumulator *Noccaea caerulescens* (Sterckeman *et al.*, 2015), *Solanum* species (Arao *et al.*, 2008; Xu *et al.*, 2012a) and soybean (Sugiyama *et al.*, 2007) that total Cd uptake depends on the genotype of the rootstock.

Consistent with the findings presented in Chapter 3, Lady Rosetta or plants with a Lady Rosetta rootstock took up more Cd than did Cara or plants with a Cara rootstock. Therefore, it was important to investigate why the root system of Lady Rosetta take up more Cd than the root system of Cara? Studies had reported that genotypic differences in root-related traits are important in explaining the difference in Cd uptake from the soil between cultivars (Lu *et al.*, 2013; Kubo *et al.*, 2016; Xia *et al.*, 2016). Examination of the root traits of the two cultivars and the selected F₁ genotypes showed that total plant Cd uptake did not show any association with root biomass or root length, as Cara, with a greater root biomass and longer roots, took up less Cd than did Lady Rosetta (Figures 4.11, 4.12). These results were consistent with previous findings from other crops, such as sunflower (Laporte *et al.*, 2015), rice (Kubo *et al.*, 2011) and peanut (Lu *et al.*, 2013).

So, if root biomass or root length are not associated with root Cd uptake, what other root trait(s) is/are associated with differences in root Cd uptake? Previous studies

examining the relationship between root systems and nutrient uptake (Fitter *et al.*, 1991; Kubo *et al.*, 2011; Lu *et al.*, 2013; Xia *et al.*, 2016) found that root volume and the number of root tips were important traits for acquisition of nutrient from the soil. Kubo *et al.* (2011) also examined the relationship between root-traits and total Cd uptake in wheat cultivars. According to the authors, a wheat cultivar with more root branches had greater total plant Cd uptake than did a cultivar with less root branches. Furthermore, root frequency (root number cm⁻² soil surface) was positively correlated with whole-plant Cd uptake. As specific root-related traits, other than root biomass and length, were not measured in the research described in this thesis, it is difficult to conclude from the existing data, whether root architecture traits (say, root branching or frequency) had any impact on Cd uptake. However, root Cd absorption “power”, as described by Nye (1973) to measure the efficiency of a genotype to take up elements including Cd, was calculated as whole-plant Cd per unit root dry weight for the two cultivars. Accordingly, Lady Rosetta had 2-fold more root Cd absorption “power” g⁻¹ DW than Cara in both grafting experiments. Results from this study were consistent with findings from the earlier study on spatio-temporal accumulation of Cd between different organs (Chapter 3), where Lady Rosetta was shown to exhibit more than 2.5-fold greater root Cd absorption power than did Cara. Further study will be needed to determine whether root-specific traits and/or any root exudates favour the greater total Cd uptake by roots of Lady Rosetta, compared to the roots of Cara.

4.4.3. Tuber Cd and Zn concentrations are largely controlled by the genotype of the scion

Despite a significant increase in Cd uptake when a Cara scion was grafted onto a Lady Rosetta rootstock, the increment to tuber Cd concentration associated with total Cd uptake was not more than 20% of tuber Cd concentration of self-grafted Cara at either soil Cd level. Conversely, plants with Lady Rosetta scions grafted onto Cara rootstock accumulated 50% and 200% greater tuber Cd concentrations than self-grafted Cara at the low and high soil Cd, respectively. These results clearly indicate that the scion is more important than plant Cd uptake (as determined by rootstock) for determining the difference in tuber Cd concentration. These results were consistent with earlier findings (Chapter 3) that the spatio-temporal accumulations of Cd and Zn between organs showed that Lady Rosetta had greater Cd and Zn concentrations in the shoot

and tuber than Cara. The results also highlighted the importance of shoot biomass for regulation of tuber Cd and Zn concentrations as a result of dilution.

Results from the selected F₁ genotypes, from a cross between these two cultivars, also revealed that tuber Cd and Zn concentrations were negatively correlated with maturity (and hence biomass). Early-maturing genotypes tended to show greater tuber Cd and Zn concentrations than late-maturing genotypes. The later-maturing genotypes had greater shoot and root biomass than did the earlier-maturing genotypes. Given that the biomass was positive correlated with maturity, and maturity affected both tuber Cd and Zn concentrations, it was logical to hypothesise that shoot biomass could influence tuber Cd and Zn concentrations through growth “dilution”.

The most important question is, why did the scion of Lady Rosetta result in greater tuber Cd concentration than did the Cara scion? As was observed in both the previous (Chapter 3) and the current studies, the Lady Rosetta scion resulted in greater shoot Cd concentration than the Cara scion. The greater shoot Cd concentration of the Lady Rosetta scion may drive greater phloem loading of Cd than by the Cara scion, which resulted in greater translocation of Cd to the tuber. The results are supported by that fact that remobilization of Cd from the shoot was shown to be the main source of Cd translocated to the tubers (Chapter 3). Furthermore, similar results have been reported in other crops, such as wheat (Clarke *et al.*, 1997) and rice (He *et al.*, 2006; Yan *et al.*, 2013), which showed that grain Cd concentration showed a positive association with shoot Cd concentration.

Further examination of the F₁ genotypes suggested that later maturity was associated with greater shoot biomass and decreased shoot and tuber Cd concentrations (Figures 4.2, 4.3, 4.4, 4.12). Kubo *et al.* (2008) reported a negative association between grain Cd concentration and agronomic traits related to shoot biomass (stem number, culm length and spikelet number per spike) in a study of 237 wheat genotypes. A study by Perrier *et al.* (2016) also reported that grain Cd concentration was negatively correlated with leaf biomass. Results from previous (Chapters 3) and current studies in this research proved that genotypic variation in shoot biomass accumulation was associated with shoot and tuber Cd concentrations. Therefore, late-maturing genotypes generally have greater shoot biomass which results in less Cd concentration in the shoot and tubers. These results were consistent with other studies in wheat (Clarke *et*

al., 1997) and rice (He *et al.*, 2006; Yan *et al.*, 2013) showing that grain Cd concentrations showed a positive association with shoot Cd concentration.

Another interesting observation in this study was that, at greater soil Cd concentrations, the scion of the genotype was the most important factor to regulate the homeostatic of Cd between shoot and root. Regardless of the source of rootstock, plants with a Lady Rosetta scion accumulated a greater Cd concentration in the root, suggesting a role for the shoot in Cd tolerance. These results are consistent with an earlier paper on the Cd-hyperaccumulator *Noccaea (Thlaspi) caerulescens*, that the genotype of the scion plays an important role in determining tolerance of an elevated zinc concentration in the soil (Guimarães *et al.*, 2009).

The story is different for Zn. Unlike Cd, leaf biomass did not show any effect on leaf Zn concentration. The data also suggested that leaf Zn concentration was negatively correlated with tuber Zn concentration. It is suggested that late-maturing potato genotypes demand more Zn for growth and development of young leaves, which act as strong sinks at the expense of the developing tubers.

The behaviour of the two transition elements was highly correlated in the stem but not in the leaves, suggesting that the shared mechanism(s) between the two transition elements might be influenced by the stem. The stems could influence the supply of Cd and Zn by favouring tuber Cd and Zn accumulation in early-maturing genotypes with greater sink strength in the early stages of growth (Trudgill & Thompson, 1987; Kooman & Rabbinge, 1996), combined with a shorter stem which minimizes trafficking distance. According to Yamaji and Ma (2014), the phloem-tropic mode, involving “preferential distribution of an element exclusively through the phloem”, is the primary distribution mechanism of Cd and Zn in rice. The mode of Cd and Zn distribution is mainly regulated by stem nodes which act as centres of trafficking. Similar results were also reported in wheat (Pearson and Rengel, 1995), where xylem-phloem-exchange operates at the rachis and, to some extent, in the peduncle, lemma and palea.

Numerous studies have proposed different mechanisms for the inter-cultivar differences in Cd levels in edible portions, including inter-cultivar differences in total Cd uptake, root-to-shoot translocation and above-ground partitioning of Cd (Jarvis *et*

al., 1976; Sugiyama *et al.*, 2007; Arao *et al.*, 2008; Xu *et al.*, 2012a; Sterckeman *et al.*, 2015). However, some factors are more important than others from a plant breeding perspective. Results from Chapters 3 and 4 showed consistency and found that the differences in tuber Cd concentrations between the two cultivars were mainly regulated by shoot biomass and, to some degree, by differences in Cd uptake and root-to-shoot translocation. On the other hand, Zn transport and distribution are highly regulated. Inter-cultivar differences in tuber Zn concentration were associated with a combination of stem dry weight and the ability of the leaves to compete for Zn with the tubers. Overall, shoot and tuber of the late-maturing genotypes may compete for Zn than early-maturing genotypes, which resulted in less Zn allocation to the tubers.

In conclusion, differences in growth habit affect both tuber Cd and Zn concentration but for different reasons. For Cd, it is a simple “dilution effect” associated with greater biomass accumulations. However, the case with respect to Zn is different; as Zn is an important micronutrient for growth and development, competition for Zn is expected between organs mainly tuber and shoot. Therefore, late-maturing genotypes need more Zn for shoot as expense of tuber than do early-maturing potato genotypes.

Chapter 5: Genetic mapping of quantitative trait loci for tuber Cd and Zn concentrations

Mengist MF, Alves S, Griffin D, Creedon J, McLaughlin MJ, Jones PW and Milbourne D (2018) Genetic mapping of quantitative trait loci for tuber-cadmium and zinc concentrations in potato reveals associations with maturity and both overlapping and independent components of genetic control. *Theoretical and Applied Genetics*, pp.1-17.

5.1.Introduction

Breeding for reduced tuber Cd concentration or greater Zn concentration in potato, as for any heritable trait, requires the availability of genetic variation for the trait to exist in breeding germplasm (Chen *et al.*, 2007; Wu *et al.*, 2015; Reuscher *et al.*, 2016).

Zn is one of the most important micronutrients for human health (White *et al.*, 2012, 2017; Kromann *et al.*, 2017). Recently, Zn biofortification has become an important objective of potato breeding programmes targeting developing countries, where micronutrient deficiency is a significant human health issue (White *et al.*, 2012, 2017; Kromann *et al.*, 2017). However, breeding for increased Zn concentration in edible plant parts may also result in increased Cd concentration in situations where soil Cd levels are problematic (White *et al.*, 2012, 2017; Kromann *et al.*, 2017).. Previous studies in cereal crops have found positive correlations between Zn and Cd in the grain of barley (Chen *et al.*, 2007; Wu *et al.*, 2015; Reuscher *et al.*, 2016), rice (Zhang *et al.*, 2011) and soybean (Sugiyama *et al.*, 2011).

Given the above observations, it seems prudent to identify both the physiological and genetic relationships between tuber Cd and Zn concentrations in potato, and to assess the potential for development of genotypes that accumulate greater Zn concentrations without simultaneously accumulating greater Cd concentrations in the tuber when it is phytoavailable in the soil. Whilst there is some information available regarding the genetic and physiological basis of tuber Zn accumulation (Subramanian, 2012), limited information is publicly available on the relationship between Cd and Zn concentrations in the tubers of potato, or any evidence of apparent genetic and physiological control of tuber Cd and Zn levels, especially in soils containing higher levels of Cd.

From a mechanistic point of view, studies have reported that differences in growth associated with maturity and biomass production may influence the distribution of elements in plants, including Cd and Zn (Kubo *et al.*, 2011; Subramanian, 2012; Yan *et al.*, 2013; Hussain *et al.*, 2016). Specifically, in potato, Subramanian (2012) reported an association between early maturity and greater tuber-Zn concentration in a segregating population, a phenomenon which was manifested both as a physical correlation between earliness and Zn concentration, and perhaps unsurprisingly, as a

co-localisation of a large effect QTL for Zn accumulation with the locus governing maturity on chromosome 5 of potato. No similar information exists for Cd in potato.

The recent development of single nucleotide polymorphism (SNP) genotyping tools such as the Infinium 8303 potato array, coupled with statistical tools that allow the incorporation of allele dosage information, offer the possibility of developing high density genetic maps with increased power for QTL detection in tetraploid potato (Felcher *et al.*, 2012; Hackett *et al.*, 2013, 2014, 2017; Massa *et al.*, 2015). Using a segregating population derived from a cross between the cultivars Cara and Lady Rosetta, this study explores the phenotypic correlation between Zn and Cd concentrations in tubers and investigates the genetic control of Zn and Cd accumulation in tubers through QTL analysis.

5.2. Materials and Methods

5.2.1. Mapping population

The mapping population was developed by crossing the two potato cultivars, with Cara as the paternal parent and Lady Rosetta as the maternal parent. The two cultivars had been extensively characterized for tuber Cd and Zn concentration in multiple experiments (Chapters 3 and 4), with Cara accumulating reduced concentrations of Cd and Zn and Lady Rosetta accumulating greater Cd and Zn concentrations. In addition, the two cultivars have contrasting agronomic characteristics. Cara is characterized by late maturity, an indeterminate growth habit, large root biomass and a tall canopy, whereas Lady Rosetta is characterized as early maturing, with a determinate growth habit, low root biomass and a short canopy. By crossing the two cultivars, 188 F₁ individuals were developed for use as a genetic mapping population.

5.2.2. Phenotyping

The F₁ mapping population, comprising tubers from each of 188 F₁ seedlings and the two parents (Cara and Lady Rosetta), were chitted and planted one tuber per pot in 15 L pots filled with soil. Details of soil characteristic and sources are described in chapter 3 (Table 3.1). The experiments were conducted in 2014 and 2015. For the 2015 experiment, soil recovered from the 2014 experiment was supplemented with additional soil collected from the same site on a 2:1 (old: new) basis. In both years, to ensure homogenous Cd and Zn distribution within each pot, the soil was air dried,

crushed and mixed very well before distribution among the pots. The soil was supplemented with 25 g NPK(S) (7-6-17(13)) fertiliser per pot. The experiment was set up as a randomized complete block design (RCBD) with three replications. Water was supplied by drip irrigation.

Upon maturity, tubers were harvested for analysis of tuber Cd and Zn concentration. The tubers were washed, peeled, and blended using a standard kitchen blender. The homogenized tuber samples were stored at -20°C until chemical analysis was carried out. The homogenized tuber sample was thawed overnight at 4°C and a sub-sample of approximately 1 g was microwave digested (MARS 5, CEM Corporation, Matthews, NC, USA) with 1 ml ultrapure water (resistivity 18 MΩcm⁻¹), 1 ml concentrated nitric acid (HNO₃, 69%, Aristar grade, VWR, Radnor, USA) and 0.5 ml hydrogen peroxide (H₂O₂, 30%, Sigma-Aldrich, Missouri, USA). The digested sample was diluted with ultrapure water and brought to a final volume of 50 ml and kept at 4°C prior to chemical analysis. Tuber Cd and Zn concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS) (Perkin Elmer ELAN DRC-e, Monza, Italy). Every digestion batch (40 samples) included two reagent blanks, a Reference Material (RM) and one sample duplicate. Reagent blanks verified the absence of contamination and were always below 0.05 and 0.2 µg ml⁻¹ for Cd and Zn, respectively. The RM used was a vegetable purée (Fapas T07188 and T07207) with a certified Cd value. Cd recoveries between 85 to 105% were obtained for the RM. Relative differences between duplicates were always less than 20%.

To express tuber Cd and Zn concentration on a dry weight (DW) basis, 5-10 g thawed homogenized tuber samples were weighed (fresh weight, FW) and oven dried at 70°C until a constant weight (dry weight, DW) was attained. DW (%) was calculated from the FW using the DW: FW ratio of the corresponding sample and multiplying it by 100. Tuber Cd and Zn concentrations were adjusted to a DW basis using their dry weight percentage. The final figures and analyses were performed using data expressed as mg kg⁻¹ DW.

The mapping population was scored for maturity in 2014 and 2015 and canopy height in 2015 in field maintenance plots of fifteen tubers without replication. The measurements followed the methodology described by Bradshaw *et al.* (2008). The maturity was scored using a 1 to 9 scale with 1 = very late maturing and 9 = very early

maturing. The canopy height was scored by taking the average measure of five plants per genotype.

Statistical analyses of phenotypic data

For QTL analysis in individual years, genotype means were calculated with simple analysis of variance (ANOVA) with genotype as a fixed factor and replication as a random factor. For analysis of variance over years (combined ANOVA) and to estimate genotypes means over years using best linear unbiased estimate (BLUE), genotype and year were considered as fixed factors.

Broad-sense heritability (H^2) was estimated using variance components calculated from the restricted maximum likelihood (REML), calculated as follows

$$H^2 = \frac{\delta_g^2}{(\delta_g^2 + (\frac{\delta_e^2}{r}))} \quad \text{and} \quad H^2 = \frac{\delta_g^2}{(\delta_g^2 + \frac{\delta_{gy}^2}{y} + (\frac{\delta_e^2}{ry}))}$$

where δ_g^2 , δ_e^2 and δ_{gy}^2 are variance components of genotypes, pot-to-pot variation of residuals and [genotype x environment] interaction, respectively; y is the number of environments (number of years in this study, =2) and r is the number of replications (=3).

Maturity effect was corrected using linear regression as described by Bradshaw *et al.* (2008) and the variance component was partitioned into components associated with maturity (mat) and maturity-corrected tuber Cd and Zn concentrations. The linear regressions are

$$\text{Cd}_{2014} = (0.0201(\text{mat}) \pm 0.003) + (0.4554 \pm 0.01694)$$

$$\text{Cd}_{2015} = (0.0179(\text{mat}) \pm 0.0021) + (0.2535 \pm 0.01190)$$

$$\text{Cd}_{\text{BLUE}} = (0.01894(\text{mat}) \pm 0.00225) + (0.3547 \pm 0.01277)$$

$$\text{Zn}_{2014} = (0.6042(\text{mat}) \pm 0.05407) + (7.7053 \pm 0.3063)$$

$$\text{Zn}_{2015} = (0.5368(\text{mat}) \pm 0.0463) + (6.8894 \pm 0.2624)$$

$$\text{Zn}_{\text{BLUE}} = (0.5708(\text{mat}) \pm 0.0043) + (7.296 \pm 0.2437)$$

5.2.3. SNP genotyping of the F₁ progeny

Approximately 3 g leaf material was collected and ground using liquid nitrogen. The fine powder was transferred to 50 mL falcon tube. Fifteen mL of CTAB (cetyltrimethylammonium bromide) buffer mix which contains 5 mL 2x CTAB (2% CTAB, 200 mM Tris pH 8, 20 mM EDTA (Ethylene diamine tetraacetic acid) pH 8, 1.4 M NaCl, 1% polyvinylpyrrolidone and 0.28% 2-Mercaptoethanol) and 10 mL of 1x CTAB buffer (1% CTAB, 200 mM Tris pH 8, 20 mM EDTA pH 8, 1.4 M NaCl, 1% polyvinylpyrrolidone and 0.28% 2-mercaptoethanol) was added to 50 mL falcon tube. The solution was mixed very well and incubated at 65°C for 1.5 hours in an oven. Afterwards, the solution was cooled down to room temperature and 10 mL of chloroform/isoamyl alcohol mix (24:1 v/v) was added and the mixture agitated continuously for 60 minutes. The mixture was centrifuged at 3000 g for 15 minutes and the upper phase was transferred slowly into a new 50 mL falcon tube using a sterile Pasteur pipette.

A second round of chloroform/isoamyl alcohol mix (24:1 v/v) was added to the newly collected supernatant at 1:1 (v/v) and agitated for 20 minutes, then centrifuged at 3500 x g for 15 minutes. The supernatant was transferred into a new 50 mL Falcon tube and then 75 µl RNase (10 mg mL⁻¹) was added, and the tube incubated at 37.5° C for 30 minutes, followed by centrifugation at 4200 x g for 15 minutes, after which the upper phase was transferred into a new 50 mL Falcon tube. Finally, DNA was precipitated by adding an equal volume of chilled (4°C) isopropanol, and the DNA was recovered by centrifugation at 3500 x g. The DNA was washed with 75% ethanol and dried for about two hours before being suspended in 500 µL TE buffer (10 mM Tris (hydroxymethyl methylamine; Sigma-Aldrich, Missouri, USA), 1 mM EDTA, pH 8). DNA quality check and estimation of DNA quantity were performed using agarose gel electrophoresis (Figure 5.1).

Single nucleotide polymorphism (SNP) genotyping was performed using the SolCap 8303 SNP array as described by Felcher *et al.* (2012). Genotyping was carried out using an Illumina iScan Reader utilizing the Infinium HD assay as a service by Gen-Probe, Manchester, UK. Genotype calling quality and preliminary data management were carried out using GenomeStudio software (version 2010.3, Illumina, San Diego, USA). Monomorphic SNPs were discarded from the data manually prior to further

analysis in GenomeStudio. From 8303 Infinium SNP markers assayed, 6287 markers were polymorphic in this initial screening.

GenomeStudio provides three clusters of genotype calling, which is similar to diploid species (homozygous for either allele or heterozygous). For autotetraploid potatoes, five alternative genotypes are possible in a full sib F₁ segregating population; nulliplex (AAAA), simplex (AAAB), duplex (AABB), triplex (ABBB) and quadruplex (BBBB). To call the genotype classes of the 6287 polymorphic SNPs, they were fitted into mixture models that use scores of overall intensity (R value) and signals of intensity (θ) using fitTetra R (Voorrips *et al.*, 2011). Data points with an R value below 0.20 were discarded. The default P threshold of 0.99, which is 99% confidence of a sample belonging to the cluster and the peak threshold of 0.85, were applied. Of the 6287 SNPs, 4557 SNPs were assigned into one of the five genotype clusters with sufficient confidence, and advanced for linkage map construction. Details of the distribution of markers by parent and dosages are presented in Table 5.1. Examples of GenomeStudio and fitTetra outputs are presented in Figure 5.2.

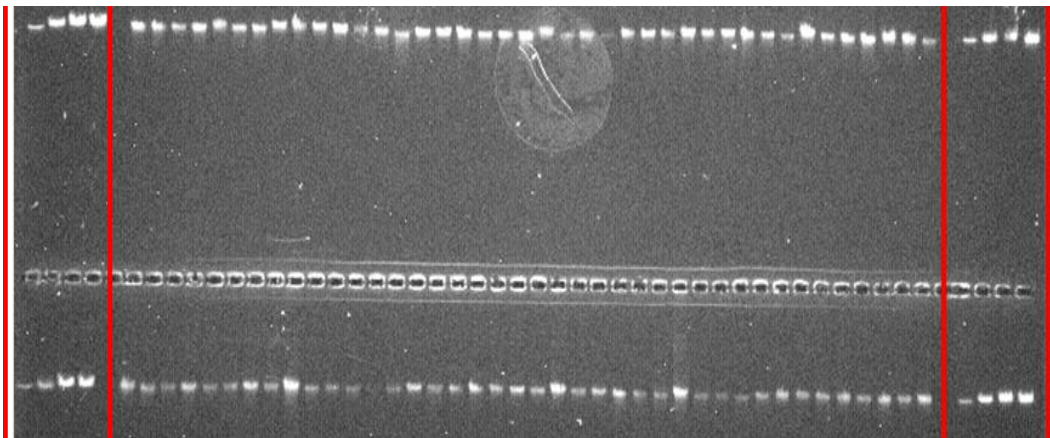


Figure 5.1 Quality check and estimation of DNA quantity using agarose gel electrophoresis. The first and the last four markers (lambda DNA) were used as standard DNA checks in ranges 50, 100, 150, and 200 ng μl^{-1} .

5.2.4. Linkage map construction

Linkage map construction was performed using TetraploidSNPMap (TPM, BioSS) software (Hackett *et al.*, 2017), following the methodology described by Hackett *et al.* (2013, 2014). First, a Chi-square test for goodness of fit was performed at $P < 0.001$

and $P < 0.01$ for simplex and higher dosage state markers, respectively. A total of 805 distorted SNPs was excluded from cluster analyses (Table 5.1).

The remaining 3752 non-distorted SNPs were clustered into 12 linkage groups using a chi-square test for independent segregation, as described by Hackett *et al.* (2013), and two-point analyses were performed for each linkage group to calculate recombination fractions and logarithm of the odds (LOD) scores. The loci were ordered using multidimensional scaling (MDS) and visual inspection of outliers (Preedy & Hackett, 2016). Once ordering was completed for each group, parental phase analyses were computed, and incomplete parental phases were manually completed. The resulting genetic map was aligned to the *Potato Genome Sequencing Consortium (PGSC) Version 4.03 Pseudomolecule* (Sharma *et al.*, 2013) and the order of markers on the genetic map was reversed if the orientation of the physical and genetic map was found to be inversely ordered in respect to the PGSC version 4.03 Pseudomolecule. Graphical presentations of linkage maps and QTLs were performed using Mapchart 2.3 (Voorrips, 2002).

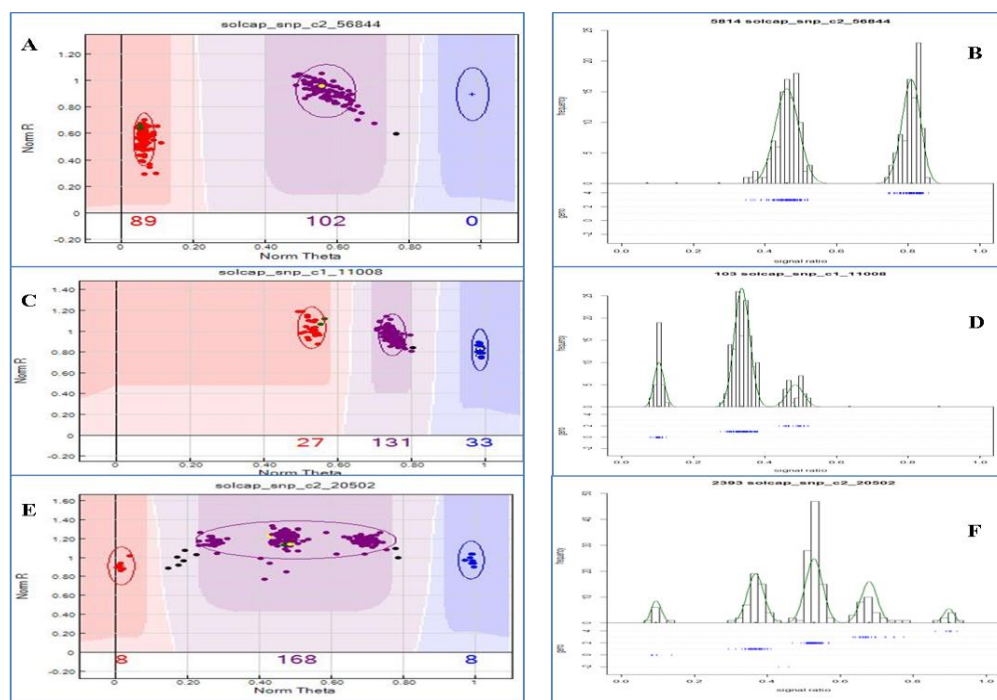


Figure 5.2 The output from GenomeStudio (left) and fitTetra (right). Displayed are examples of different dosage types, simplex by nulliplex (A, B; 1:1), duplex by nulliplex (C, D; 1:4:1) and double duplex (E, F; 1:8:18:8:1).

5.2.5. QTL mapping

QTL analysis was performed using TetraploidSNPMap for each trait, considering markers from both parents (Hackett *et al.*, 2017). The phenotypic data were regressed to the QTL genotypes at each position, with the regression coefficients being weighted by the conditional probabilities of the QTL genotypes. The trait value was modelled as an additive function of the QTL allele effect on each of the eight homologous chromosomes (referred to in the results as the “full model”) as described in Hackett *et al.* (2014). The full model is an additive function of the QTL allele effect on each of the eight homologous chromosomes for each position on the grid. This model is fitted by regression of the trait values on the QTL genotype probabilities from the Hidden Markov Model (HMM).

For each trait, a significant QTL was declared with 200 permutations and 95% confidence interval thresholds. For a significant QTL, the “simpler model” function was run and the best simple model was identified using the Schwarz Information Criteria (SIC) (Schwarz, 1978). The SIC is calculated in TPM as follows:

$$\text{SIC} = -2\log L + p \log m_o,$$

where L is the likelihood of the simple model, p is the number of parameters in the simple model and m_o is the number of observations (the 36 genotype means). The best simple model is the lowest value for the SIC and the best simple model is considered a good fit if the SIC is lower than that of the full model, and the adjusted R^2 values of the simple model are close to or better than the full model (Hackett *et al.* 2014). For presentation, homologous chromosomes derived from Lady Rosetta are designated h1 to h4 and those derived from Cara are designated h5 to h8.

In cases where two closely linked QTLs were identified on the same chromosome, the version of TetraploidSNPMap used for analysis could not estimate the individual QTL LOD and parameter values, yielding data only pertaining to the largest effect. To estimate LOD and parameter values of the second QTL, the SNPs closest to the largest peak with the configuration of the best SIC model was selected and then the phenotypic data of the trait were regressed on the selected SNP (Christine Hackett, personal communication). The residuals from this fitted model were used as the phenotypic data to estimate the LOD and other parameter values of the second QTL.

If the second QTL was not significant after this process, I considered the two QTLs to be a single effect.

Table 5.1 Distribution of SNPs into genotype classes

Marker class	Cara	LR	Segregation ratio	Total SNPs	Distorted SNP	Non-distorted SNPs
Simplex from Cara	AAAB	AAAA	1:1	920	25	895
Simplex from LR	AAAA	AAAB	1:1	661	28	633
Triplex from Cara	ABBB	AAAA	1:1	28	8	20
Triplex from LR	AAAA	ABBB	1:1	24	4	20
Duplex from Cara	AABB	AAAA	1:4:1	149	34	115
Duplex from LR	AAAA	AABB	1:4:1	207	54	153
Double-simplex	AAAB	AAAB	1:2:1	719	77	642
Simplex-duplex	AAAB	AABB	1:5:5:1	652	184	468
Duplex-simplex	AABB	AAAB	1:5:5:1	585	162	423
Simplex-triplex	AAAB	ABBB	1:2:1	126	47	79
Triplex-simplex	ABBB	AAAB	1:2:1	85	31	54
Double-duplex	AABB	AABB	1:8:18: 8:1	401	151	250
Total				4557	805	3752

Significant distortion at $P < 0.001$ and $P < 0.01$ for simplex and higher dosage markers, respectively; LR=Lady Rosetta.

5.3.Results

5.3.1. Phenotypic evaluation of mapping population

As expected, the average (over the two years) tuber Cd and Zn concentrations were greater in Lady Rosetta (0.910, 14.41 mg kg⁻¹ respectively) than Cara (0.452, 10.38 mg kg⁻¹ respectively) (Table 5.2). The mean values of tuber Cd and Zn concentration in the two parents were less in 2015 than 2014 (Table 5.2). The analysis of variance showed significant variation for genotype, year and [genotype x year] interaction (Appendix-Table 8.7). The 2-year broad-sense heritability for tuber Cd and Zn concentrations were quite large (≥ 0.77) for individual years and over years (Table 5.2).

Extensive genetic variation was observed in the segregating population for both traits in both years, with an approximately 3-fold range of variation observed for both tuber Cd and Zn concentrations (Table 5.2). In 2014, the mean value of tuber Cd concentration in the segregating population was 0.56 mg kg⁻¹ which was larger than

the mean value of tuber Cd concentration in 2015, 0.35 mg kg⁻¹ (Table 5.2). With respect to tuber Zn concentration, the mean value of the mapping population was similar between the two years, namely 10.79 mg kg⁻¹ in 2014 and 9.63 mg kg⁻¹ in 2015 (Table 5.2).

Tuber Cd and Zn concentrations both exhibited a near-normal distribution in the population in both years, suggesting polygenic control of the traits (Figure 5.3). The mean value of the concentration for both transition elements in the population was less than the mean value of the parents in both years (2014, 2015), suggesting one-directional transgressive segregation (Table 5.2, Figure 5.3).

5.3.2. Correlations between Cd, Zn concentration and growth-related traits

There was a significant ($P < 0.01$) positive correlation between tuber Cd and Zn concentration in the population in both years (Table 5.3). Both tuber Cd and Zn concentration also exhibited significant ($P < 0.01$) correlations with canopy height and maturity. Early- maturing genotypes and those with a short canopy height tended to accumulate greater tuber Cd and Zn than late-maturity genotypes and those with a taller canopy height (Table 5.3). In both years and for both traits, Zn was more strongly correlated with maturity than was Cd (Figure 5.3).

Table 5.2 Parents and F₁ population means and broad-sense heritability (H²)

Trait	Parent Mean		Parents average	Population mean	Population	
	Cara	LR			range	H ²
Cd_2014	0.452	0.910	0.681	0.558	0.304-0.920	0.81
Cd_2015	0.269	0.531	0.400	0.345	0.191-0.732	0.88
Cd over years	0.361	0.720	0.541	0.451	0.280-0.749	0.77
Zn_2014	10.382	14.406	12.394	10.792	6.303-18.425	0.88
Zn_2105	8.951	13.102	11.027	9.632	6.403-15.841	0.84
Zn over years	9.667	13.754	11.710	10.212	6.665-15.589	0.81

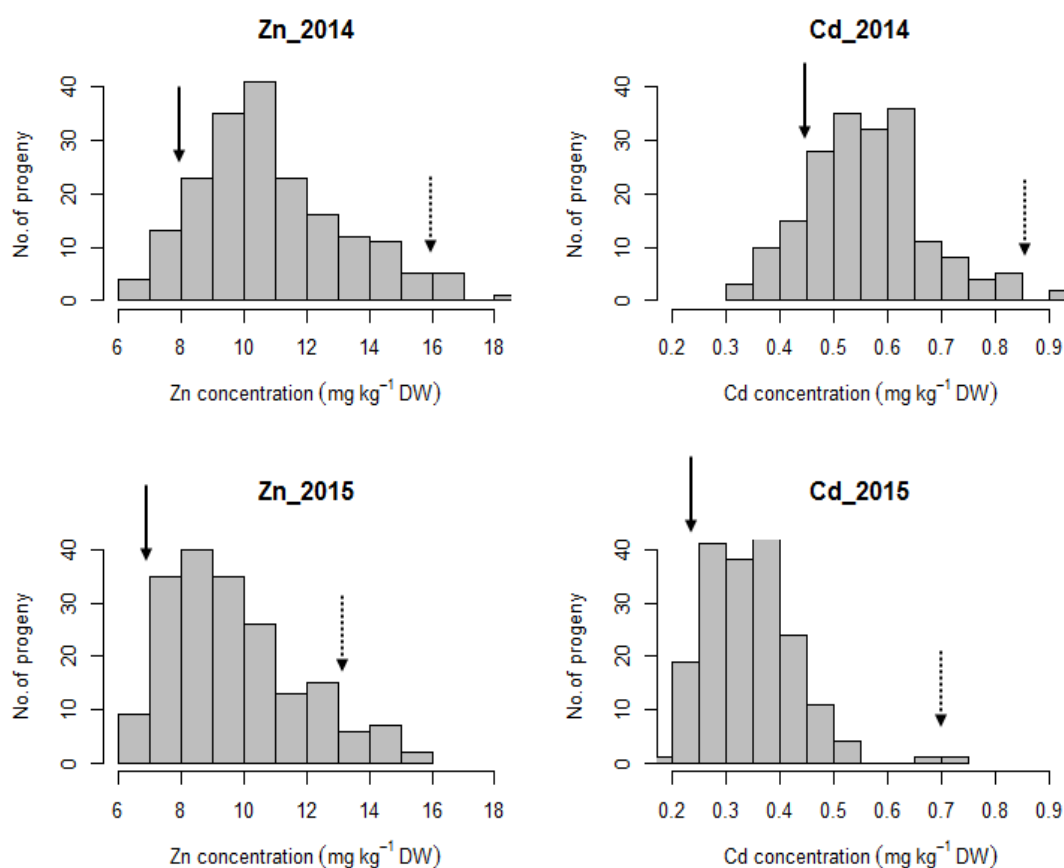


Figure 5.3 Frequency distribution of tuber Cd and Zn concentration of 188 F₁ mapping population, derived from Cara and Lady Rosetta. Parent positions are marked by a broken arrow (Lady Rosetta) and bold arrow (Cara).

Table 5.3 Pearson coefficients of correlation within 188 F₁ mapping population

2014 Year				2015 Year		
	Cd	Zn	Maturity	Cd	Zn	Maturity
Cd	1			1		
Zn	0.54**			0.61**		
Maturity	0.44**	0.63**		0.53**	0.65**	
CP	-0.26**	-0.51**	-0.69**	-0.35**	-0.57**	-0.69**

Maturity, 1 to 9 (1=very late, 9=very early); CP, plant canopy height (cm); tuber Zn and Cd concentration (mg kg⁻¹ DW); ** significant at $P < 0.01$.

5.3.3. Genotyping and linkage map construction

A total of 3752 (45%) of the markers assayed were available for linkage map construction, consisting of 1528 simplex SNPs (AAAB x AAAA), 268 duplex SNPs (AABB x AAAA), 642 double simplex SNP (AAAB x AAAB) and 1314 higher

dosage markers. The proportion of simplex markers to the total markers in each linkage map varied from 31% on chromosome 8 to 57% on chromosome 11. The three marker types (simplex by nulliplex, duplex by nulliplex and double simplex) comprised 58 % to 75% of the total markers per linkage group. Previous versions of TetraploidMap could only incorporate these three marker classes efficiently into the map; thus, a considerable increase in marker density was possible using the new version (TetraploidSNPMap) (Appendix-Table 8.8).

From 3752 SNPs, 821 SNPs were duplicates or near-duplicates (differing for at most two of the 190 genotypes), which were filtered out during two-point analysis while another 38 SNPs were identified as outliers during multi-dimensional scaling (MDS) analysis, were also removed. Details of the 2D MDS analysis for each chromosome are presented (Appendix-Figure 8.8). The final map comprised 2893 SNP markers covering 985.7 cM (Appendix-Figure 8.9), with an average inter-locus distance of 0.36 or 2.94 markers per cM. The average length of a linkage group was 82.14 cM, ranging from 70.90 cM on chromosome 10 to 105.60 cM on chromosome 1. The average inter-locus distance varied from 0.28 on chromosomes 6 and 7 to 0.65 on chromosome 12 (Table 5.4). Whilst marker coverage/density on the combined map (Figure 5.4) was generally quite high, there were intervals that were poorly covered. This is more obvious when looking at the coverage of the individual homologues as presented in Appendix-Figure 8.9, where individual gaps approaching 30 cM are apparent (eg. on h8 of chromosome XII).

Alignment of the integrated map (cM) with the physical map (Mb) PGSC version 4.03 Pseudomolecule of the reference genome revealed generally good agreement in terms of marker order (Appendix-Figure 8.10). On the parental basis, the overall combined genetic map covered almost the full length of the physical map for all chromosomes (Figure 5.4, Table 5.4).

5.3.4. QTL analysis of tubers Cd and Zn concentration

Tuber Cd concentration

A major-effect QTL was detected on chromosome 5 with LOD scores of 15 and 16 for 2014 and 2015 respectively. For each year, this QTL explained approximately 27% of the phenotypic variance and was associated with greater tuber Cd concentration.

When using the joint analysis of two years' data as input for the analysis (BLUE analysis), the trait variance explained by this QTL increased to 33%. Analysis of the QTL genotype means using the simple model showed that a simplex allele on homologue 1 (h1) of Lady Rosetta had the lowest SIC compared to the SIC of the full (additive) model (Table 5.5).

The closest SNP marker with simplex configuration was *solcap_snp_c2_11829* (PGSC0003DMG400030549) at 25 cM on chromosome 5. This is ~0.50 Mb from the *CDF1* gene (PGSC0003DMG400018408, chr5:4538880-4541736) coding for variation in plant maturity (Kloosterman *et al*, 2013). Moreover, a minor-effect simplex allele on homologue 5 (h5) of Cara showed an additive effect with the simplex allele on h1 from Lady Rosetta. The closest SNP associated with h5 of Cara was *solcap_snp_c2_38163* (PGSC0003DMG400022606) (Table 5.5, Figure 5.5).

Only one further QTL for tuber Cd concentration was found to be significant across both years, this QTL was on chromosome 6 with a peak LOD score of 6.95 and 4.24 for 2014 and 2015, respectively, and explained 11 and 6 % of the phenotypic variance for 2014 and 2015 respectively. When BLUE analysis data were used, this QTL explained 10% of the phenotypic variance (Table 5.5, Figure 5.5). Analysis of the QTL genotype means using the simple model showed that a simplex allele on homologue 2 (h2) of Lady Rosetta had the lowest SIC compared to the SIC of the full (additive) model (Table 5.5). The closest marker with simplex configuration was *solcap_snp_c1_11139* (PGSC0003DMB000000575) at 62.56 cM on chromosome 6. Interestingly, despite being derived from the parent accumulating greater concentrations of tuber Cd, this QTL reduced tuber Cd concentration by 8% when present (Table 5.5).

Two very closely linked minor-effect QTLs were detected only in one year (2014), on chromosome 3. It was not able to distinguish the effects using the process described in Materials and Methods and, for the purposes of the study, describe this as a single effect. The highest peak possessed a LOD score of 5 and explained 8.5% of the phenotypic variance. When the BLUE values were used as phenotypic data, this QTL was detected with a LOD of 4.62 and explained 7% of the phenotypic variance (Table 5.5, Figure 5.5). Inspection of the QTL analysis output for 2015 data indicated the presence of an effect exceeding a LOD threshold of 3 (3.06) in a similar position as

the QTLs from the 2014 or BLUE data, but falling below the cut-off threshold established by the permutation analysis, suggesting that the effect was present in both years (Table 5.5, Figure 5.5).

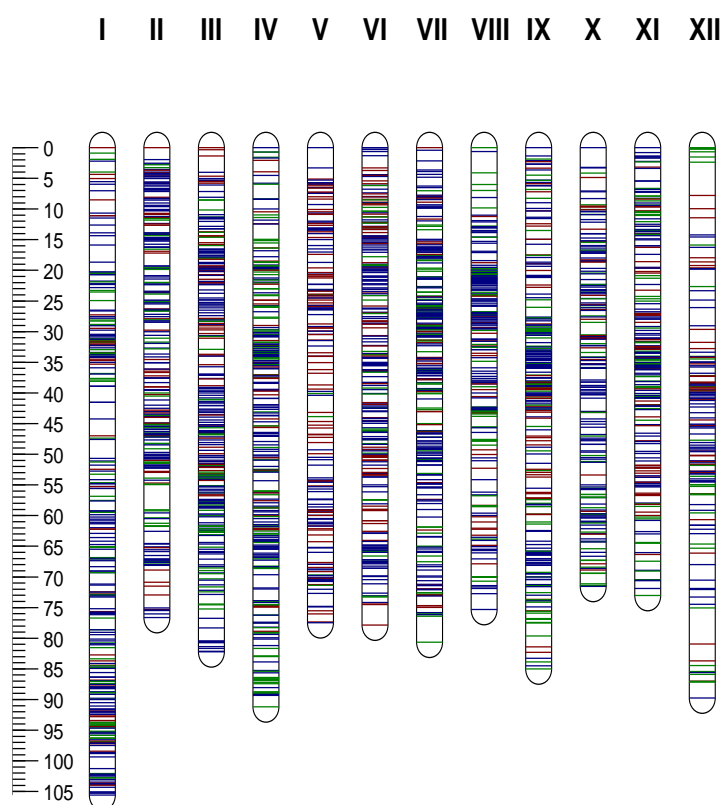


Figure 5.4 Distribution of SNP markers on 12 chromosomes (I–XII). SNP positions are marked in green (from “Cara”), dark red (from “Lady Rosetta”) and blue (common markers). The scale shows the genetic distance in cM.

Maturity-correction of tuber Cd concentration identified a further QTL on chromosome 7. This effect was detected in both individual years, and the BLUE-derived data. This QTL explained 7.5 and 9.2% of the phenotypic variance for 2014 and 2015, respectively. The phenotypic variance explained by this QTL was improved to 10% when the joint analyses of two years’ data were used. The simple model analysis showed that the simplex marker associated with increased tuber Cd concentration was located on homologue h1 of Lady Rosetta.

In addition to allowing the detection of a novel QTL, maturity correction also increased the significance of previously detected QTLs on chromosomes 3 and 6 (Figure 5.5). The “double peak” QTL on chromosome 3 was detected as a single effect spanning the confidence interval covered by the two peaks (Figure 5.5), and this QTL had a higher LOD (5.4, 4.89) and phenotypic variance levels (9% and 8%) for the 2014 and BLUE-derived data, respectively. Similarly, the QTL effect on chromosome 6 increased to explain 12.4 % of the phenotypic variance. Unsurprisingly, the QTL

effect on chromosome 5 was reduced to 8%, indicating that maturity correction was reasonably effective (Table 5.5, Figure 5.5).

Tuber Zn concentration

For tuber Zn concentration, a major-effect QTL was located on chromosome 5, at the same location as that of the largest tuber Cd concentration QTL. This explained 33, 31% and 38% of phenotypic variance for 2014, 2015 and when BLUE-derived data were used, respectively. When the tuber Zn concentration values were predicted using two years' data, this QTL explained 38% of the phenotypic variance (Table 5.5, Figure 5.5). As observed for tuber Cd concentration, the simplex SNP, *solcap_snp_c2_11829*, was also the closest SNP for tuber Zn concentration and regression of tuber Zn concentration values on the genotypes of this SNP explained 26% and 25% of the trait variance for 2014 and 2015, respectively. When the BLUE data were regressed on this SNP, the trait explained 30% of the phenotypic variance.

A smaller-effect QTL for tuber Zn concentration was identified on chromosome 3 for 2015 with a LOD of 4.02, which explained 5% of the phenotypic variance. The QTL effect on chromosome 3 was also detected with a LOD of 3.77 and 5% of the phenotypic variance using BLUE derived data (Table 5.5, Figure 5.5).

Similarly, two distinct smaller-effect QTLs on chromosome 6 were detected for 2014 and when BLUE-derived data were used. Detailed analysis of the full model showed that the highest peak on chromosome 6 was recorded at 45 cM with LOD of 4.26 and phenotypic variance of 7% for 2014 and LOD of 3.93 and 6% phenotypic variance when the BLUE data were used. Simple model analysis also showed that this QTL had a positive effect on Zn concentration (Figure 5.5, Table 5.5). The best simple model was associated with h4 from Lady Rosetta, although no simplex marker is present near the QTL peak. The second-best simple model was associated with the double simplex marker *solcap_snp_c2_33830*, which is the closest marker to the peak of the QTL. TetraploidSNPMap did not report the variation explained by a distinct second peak for Zn, located in the same position as the QTL for tuber Cd concentration at 71-76 cM on chromosome 6 (Figure 5.5).

To estimate the effect of this second QTL, *solcap_snp_c2_33830*, was regressed on the phenotypic datasets. The residuals from the fitted data were applied for QTL

analysis. Using this approach, QTLs were detected at 73 cM with LOD 5.08 and 5.19 for maturity corrected 2014 and BLUE-derived data, respectively. This QTL had the same negative effect on Zn concentration as the co-localised Cd QTL had for tuber Cd concentration, and explained ca. 7% of the phenotypic variance (Table 5.5, Figure 5.5). Thus, the QTL for tuber Zn concentration at 73 cM corresponds with a QTL for tuber–Cd concentration resulting in a reduction in both tuber Cd and Zn concentration, while the QTL at 42 cM is independent of tuber Cd concentration and associated with increased tuber Zn concentration.

Maturity-corrected tuber Zn concentration values allowed the detection of a further QTL on chromosome 1 with a LOD of 4.2, which explained 6.4 and 7% of the phenotypic variance respectively in 2015 and BLUE-derived phenotypic data, respectively. As expected, the QTL effect on chromosome 5 was reduced when maturity corrected data were applied (7% of phenotypic variance explained) (Table 5.5, Figure 5.5). In summary, among the five QTLs identified for tuber Zn concentration, three loci, on chromosomes 3, 5 and 6, coincided with those for tuber Cd concentration, sharing the direction of effect in all cases.

5.3.5. QTLs for maturity and canopy height

For maturity and canopy height, the well-established major-effect QTL on chromosome 5 (Bradshaw *et al.*, 2004, 2008; Kloosterman *et al.*, 2013; Hackett *et al.*, 2014) was detected in the population. This coincided with the QTLs for tuber Cd and Zn concentration, explaining 53% and 35% of the phenotypic variance for maturity and canopy height respectively. Similar to tuber Cd and Zn concentration, the *solcap_snp_c2_11829* was the closest marker with simplex configuration associated with earliness and short canopy height. When the phenotypic values of maturity and canopy height were regressed on the genotypes of this SNP, it explained 45% and 27 % of the phenotypic variance for maturity and canopy height, respectively.

In addition, a QTL was detected on chromosome 4 which affected maturity and canopy height with a peak LOD score of 5.25 and 4.92 respectively, and explained 10% and 9% of the phenotypic variance for maturity and canopy height, respectively (Table 5.5, Figure 5.5). The simple model analysis showed a QTL with double simplex marker with additive allelic effects on h3 and h8 of Lady Rosetta and Cara respectively. The double simplex SNP *solcap_snp_c2_48691*

(PGSC0003DMG400012800) explained 8% of the phenotypic variance and was associated with early maturity or short canopy height. Furthermore, two minor-effect QTLs on chromosomes 9 and 11 were also detected with effect on canopy height, which each explained ca. 8% of the phenotypic variance (Table 5.5, Figure 5.5). However, only the major-effects QTL on chromosome 5 was co-localized with a QTL for tuber Cd and Zn concentration. Details of the QTL on linkage maps of the haplotypes of the two parents are presented (Appendix-Figure 8.11).

Table 5.4 Summary of integrated linkage map

Chr.	SNPs	Outliers	mappable		SNPs mapped	Map length (cM)	Map length (Mb)		PGSCv4.03 PM (Mb)	Map Coverage		Average inter-loci distance (cM)
			SNPs	Duplicates			Cara	LR		Cara	LR	
1	358	1	357	79	278	105.6	88.47	88.58	88.58	1.0	1.00	0.38
2	311	5	306	52	254	76.6	47.63	45.70	48.56	0.98	0.94	0.30
3	352	1	351	80	271	82.2	62.03	62.03	62.27	1.00	1.00	0.30
4	390	2	388	84	304	91.2	72.03	71.95	72.04	1.00	1.00	0.30
5	221	4	217	39	178	77.6	52.00	52.00	52.00	1.00	1.00	0.42
6	355	1	354	74	280	77.8	59.36	59.36	59.36	1.00	1.00	0.28
7	389	2	387	97	290	80.6	56.63	56.63	56.63	1.00	1.00	0.28
8	315	12	303	73	230	75.3	56.78	56.78	56.79	1.00	1.00	0.33
9	323	2	325	76	249	85.0	61.47	61.47	61.47	1.00	1.00	0.34
10	238	0	238	49	189	70.9	59.66	59.26	59.66	1.00	0.99	0.38
11	313	7	306	76	230	73.1	45.13	45.09	45.42	0.99	0.99	0.32
12	183	1	182	42	140	89.8	61.14	61.09	61.15	1.00	1.00	0.65
Total	3752	38	3714	821	2893	985.7	722.44	719.94	723.93	1.00	0.99	0.36

LR, Lady Rosetta; SNP, Single Nucleotide Polymorphism; cM, centimorgan; Mb, Megabase; Map length (Mb) and map coverage values are based on the PGSC version 4.03 Pseudomolecules of the reference *Solanum tuberosum* group Phureja DM1-3 516 R44 (DM), Outliers were identified using multi-dimensional scaling (MDS) analysis.

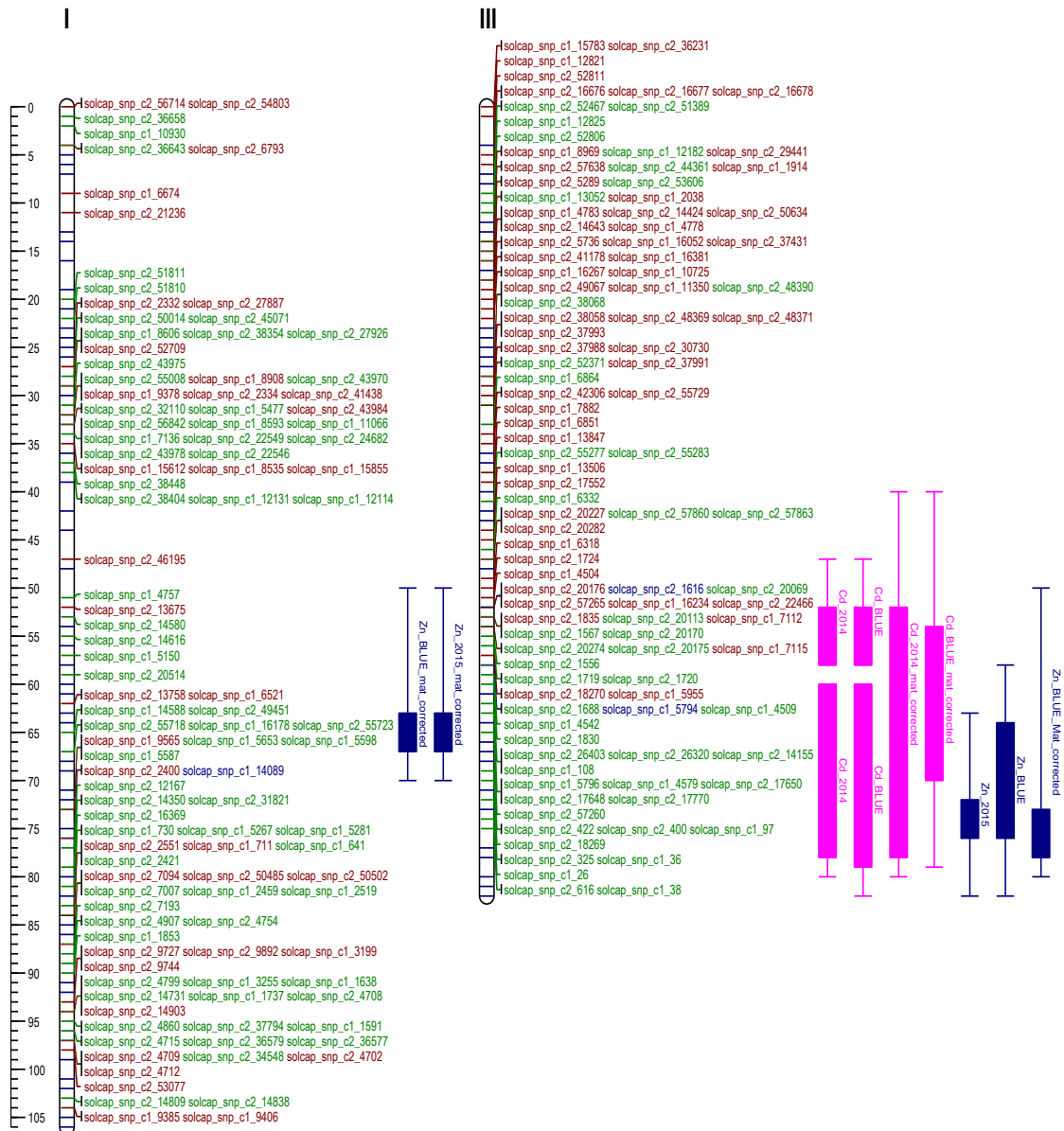
Table 5.5 Summary of quantitative trait loci for tuber Cd and Zn concentration and agronomic traits in the mapping population

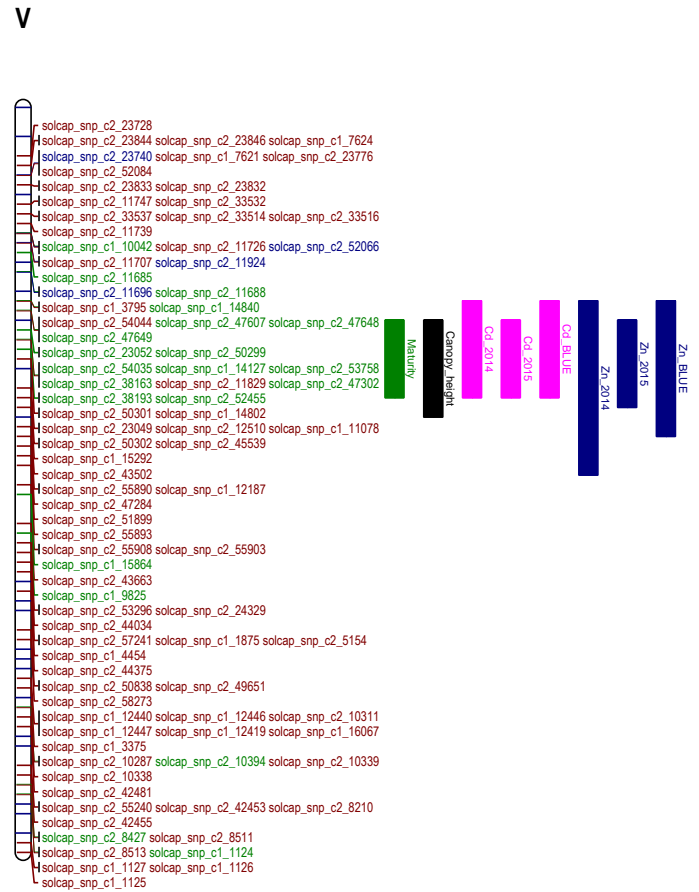
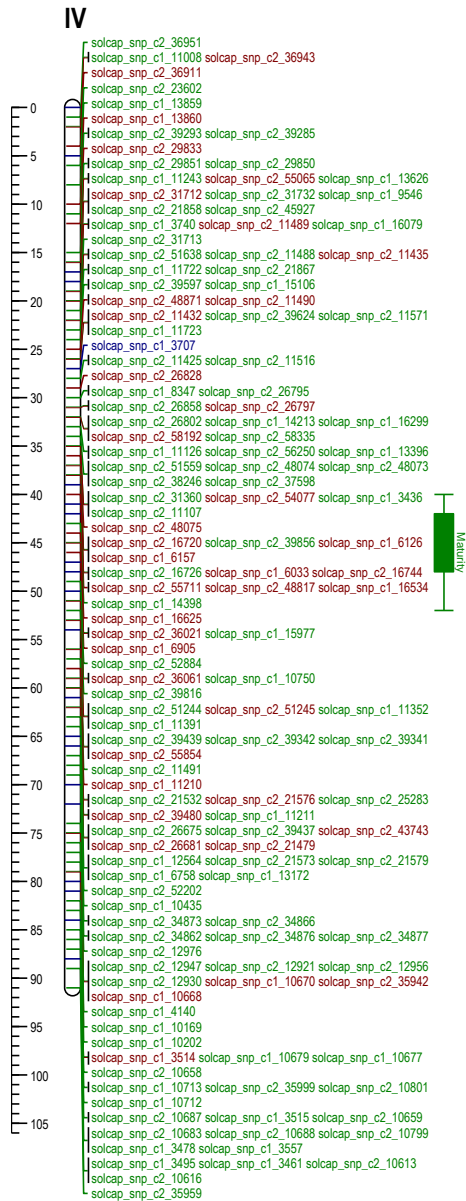
Trait	Chr.	LOD (95% CI)	R ²	Simple model and SNP (if identified)	<u>Allelic substitution</u>		
					Present	Absent	Effect
Cd_2014	3	5.12 (47-58,60-79)	8.5	DS on h2 and h7, solcap_snp_c2_323	-	-	+ve
Cd_BLUE	3	4.62 (52-58,61-70)	7.0	As for Cd_2014	-	-	+ve
Cd_2014_mat_corrected	3	5.41 (53-70)	9.0	As for Cd_2014	-	-	+ve
Cd_BLUE_mat_corrected	3	4.90 (53-70)	8.0	As for Cd_2014	-	-	+ve
Cd_2014	5	15.20 (22-28)	27.0	Simplex on h1, solcap_snp_c2_11829	0.60	0.51	0.09
Cd_2015	5	16.00 (24-28)	28.0	As for 2014	0.38	0.31	0.07
Cd_BLUE	5	19.00 (22-28)	33.0	As for Cd_2014	0.49	0.41	0.08
Cd_2014	6	6.95 (68-77)	11.0	Simplex on h2, solcap_snp_c1_11139	0.53	0.59	-0.06
Cd_2015	6	4.24 (69-77)	6.0	As for Cd_2014	0.33	0.36	-0.03
Cd_BLUE	6	6.62 (68-77)	10.0	As for Cd_2014	0.43	0.47	-0.04
Cd_2014_mat_corrected	6	7.73 (59-77)	12.0	As for Cd_2014	-0.03	0.03	-0.06
Cd_2015_mat_corrected	6	4.25 (70-77)	6.0	As for Cd_2014	-0.01	0.01	-0.02
Cd_BLUE_mat_corrected	6	7.71 (62-77)	12.5	As for Cd_2014	-0.02	0.02	-0.04
Cd_2014_mat_corrected	7	4.69 (35-55)	7.5	Simplex on h1, solcap_snp_c1_3153	0.02	-0.02	0.04
Cd_2015_mat_corrected	7	5.34 (28-42)	9.2	Simplex on h1, solcap_snp_c1_3153	0.02	-0.02	0.04
Cd_BLUE_mat_corrected	7	5.50 (29-46)	10.0	simplex on h1, solcap_snp_c1_3153	0.02	-0.02	0.04
Zn_2015_mat_corrected	1	4.25 (63-67)	6.4	additive duplex on h5 and h7	-	-	+ve
Zn_BLUE_mat_corrected	1	4.20 (64-67)	7.0	additive duplex on h5 and h7	-	-	+ve
Zn_2015	3	4.02 (73-76)	5.0	DS on h4 and h5, solcap_snp_c2_323	-	-	+ve
Zn_BLUE	3	3.77 (64-76)	5.0	As for Zn_2015	-	-	+ve
Zn_BLUE_mat_corrected	3	4.09 (72-76)	5.3	DS, h2 and h7	-	-	+ve

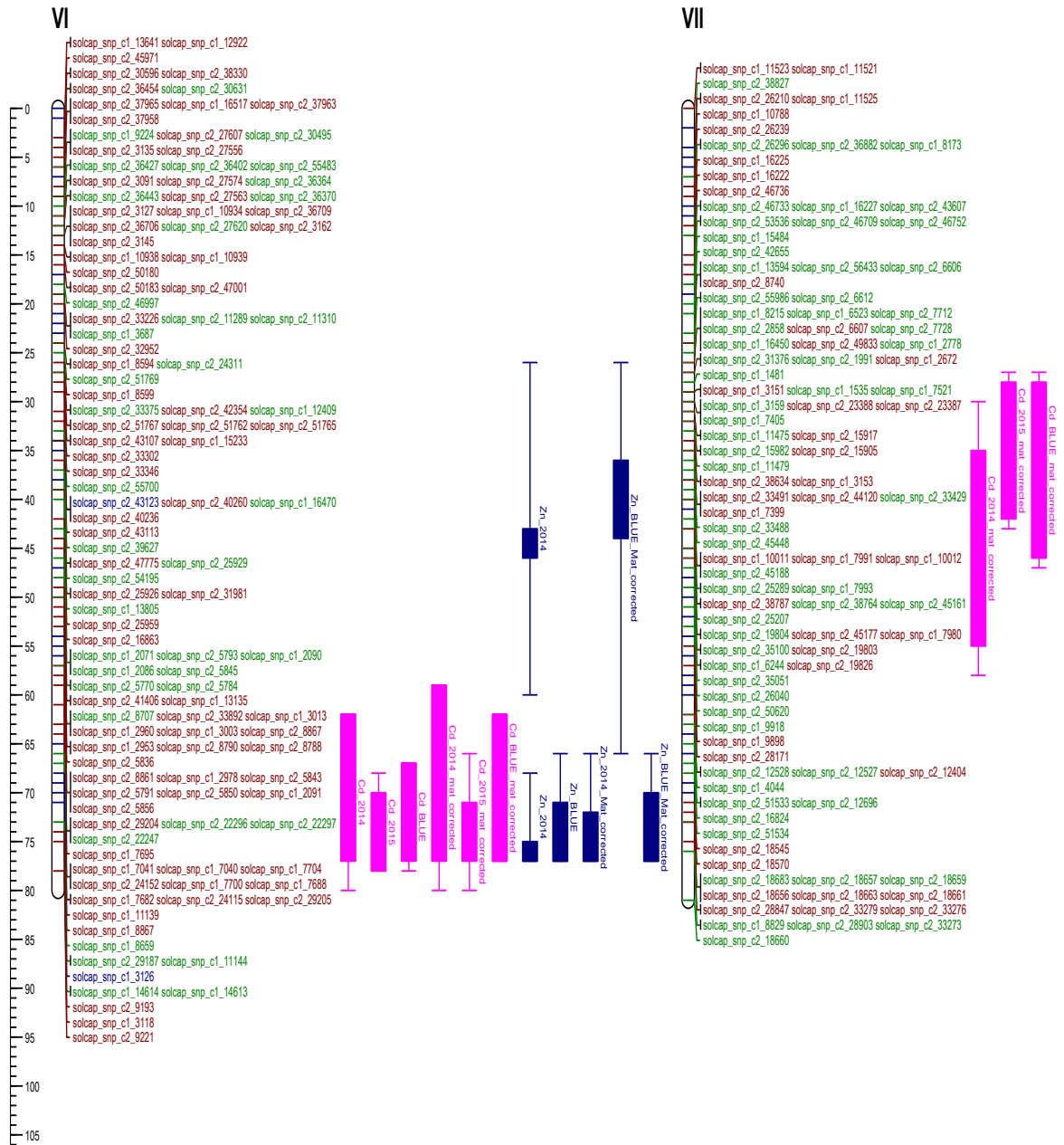
Table 5.5 Summary of quantitative trait loci for tuber Cd and Zn concentration and agronomic traits in the mapping population

Trait	Chr.	LOD (95% CI)	R ²	Simple model and SNP (if identified)	<u>Allelic substitution</u>		
					Present	Absent	Effect
Zn_2014	5	19.00(22-40)	33.0	Simplex on h1, solcap_snp_c2_11829	11.93	9.55	2.38
Zn_2015	5	17.60 (24-29)	31.0	Simplex on h1, solcap_snp_c2_11829	10.60	8.58	2.02
Zn_BLUE	5	22.00 (22-32)	38.0	Simplex, solcap_snp_c2_11829	11.27	9.07	2.20
Zn_2014	6	4.26 (42-46)	7.0	Simplex h4	10.40	11.22	-0.82
Zn_BLUE	6	4.02 (70-77)	6.0	V12	9.92	10.53	-0.61
Zn_2014_mat_corrected	6	5.08 (71-76)	6.3	V12	-0.40	0.43	-0.83
Zn_BLUE_mat_corrected	6	5.19 (34-44,70-77)	7.0	V12	-0.30	0.32	-0.62
Mat	4	5.32 (42-48)	8.7	DS on h3 and h8, solcap_snp_c2_48691	-	-	-ve
Mat	5	36.00 (24-28)	53.0	Simplex on h1, solcap_snp_c2_11829	6.68	3.4	3.28
CP	4	4.92 (53-63)	8.3	As for maturity	-	-	-ve
CP	5	22.00 (24-30)	37.0	As for maturity	79.03	98.03	-19.00
CP	9	4.83 (33-37)	8.0	DS on h2 and h7	-	-	-ve
CP	11	5.26 (53-65)	7.5	DS on h2 and h8	-	-	+ve

Chr., chromosome number; LOD, threshold values of logarithms of odds (LOD) for the QTL calculated by TetraploidSNPMap; 95% CI, the 95% support LOD interval; R², phenotypic variance explained by the QTL; BLUE, best linear unbiased estimate; CP, plant canopy height; DS, double simplex marker; V12, double duplex as codominant variate on homologous chromosome 1 and 2; Simplex models are indicated as homologous chromosomes h1-h4 from Lady Rosetta and h5-h8 from Cara. Allelic substitution effect was performed on those QTLs with the best simplex model. For higher dosage SNP, only direction of the effect is presented. The effects are presented 1 to 9 (1 =very late, 9 =very early) for maturity; CP (cm) and Cd and Zn concentration ($\mu\text{g g}^{-1}$).







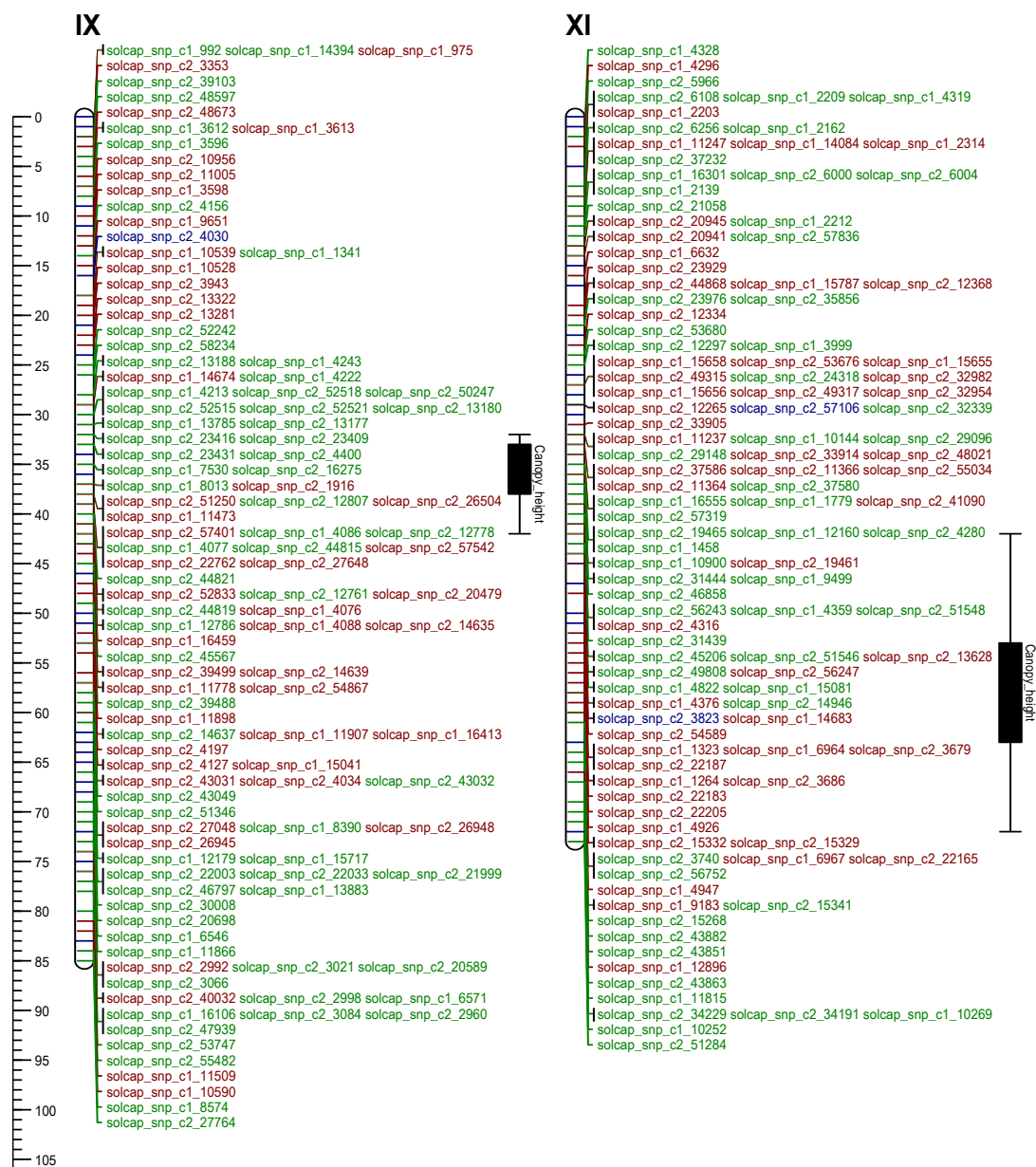


Figure 5.5 The boxplot represents the QTL position for Cd (rose), Zn (blue), maturity (green), and canopy height (black) of the chromosome. In the boxplot, whiskers correspond to the two LOD support interval and the solid box represents the 95% support LOD interval. For chromosome V, the 95% support LOD interval covered a large part of the chromosome. For simplicity, only two LOD support intervals are presented for chromosome V for all traits. SNPs positions are marked in green (from Cara), dark red (from Lady Rosetta) and blue (common markers). The scale shows the genetic distance in cM.

5.4. Discussion

This is the first report to provide an insight into the relationships between tuber Cd and Zn concentrations and agronomic characteristics, using a genetic mapping approach in potato. This approach allowed examination of the relationship between tuber Zn and Cd concentrations on a phenotypic level, and dissection of the genetic control of tuber accumulation of both transitional elements in the cross.

In this study, the individuals of the mapping population showed ca. 3-fold variation for both tuber Cd and Zn concentration. This is consistent with previous studies for the magnitude of variation for tuber Cd (McLaughlin *et al.*, 1994, 1997; Dunbar *et al.*, 2003) and Zn concentration (Andre *et al.*, 2007; Burgos *et al.*, 2007; Brown *et al.*, 2011; Abebe *et al.*, 2012; Subramanian, 2012; White *et al.*, 2017). On a phenotypic level within the population, Cd and Zn concentration in the tuber were positively correlated, and both were negatively associated with maturity, with the early phenotype being associated with an increased accumulation of both transitional elements in tubers. An association between earliness and tuber Zn concentration in a segregating potato population of biparental origin is in line with previous observations by Subramanian (2012). However, little published information exists on the association between Cd concentration and maturity. Cara and Lady Rosetta were chosen as the parents of this mapping population on the basis that they represented the extremes in Cd accumulation from a survey of ~14 varieties over a number of years at Teagasc (Alves *et al.* in preparation). Whilst Cara and Lady Rosetta represent divergent maturity classes, it is worth noting that, in the aforementioned survey of 14 varieties, the association between tuber Cd concentration and maturity was not clear (data not shown). Even within the current population, it was observed that individual genotypes exhibiting large tuber Cd and Zn concentrations also exhibited late maturity, and vice versa (Appendix-Figure 8.12). Thus, from the phenotypic data alone, it is possible to conclude that maturity certainly has a strong influence on tuber Cd and Zn concentration, but that the relationship is not absolute, and other unrelated mechanisms must also be involved in the accumulation of transitional elements in the tubers.

5.4.1. QTLs for tuber Cd and Zn concentrations

QTL analysis from these two years' study identified four loci for tuber Cd concentration on chromosomes 3, 5, 6 and 7, which explained phenotypic variation ranging from 5 to 33%. Similarly, five QTLs were identified for tuber Zn concentration on chromosomes 1, 3, 5 and 6, which explained 5 to 38% of the phenotypic variance. Unsurprisingly, given the strong correlation between maturity and both element traits, the largest effect QTL for both tuber Zn and Cd concentration was found to coincide with the maturity locus on chromosome 5 (Figure 5.5). The largest detectable effect associated with simplex QTL allele from Lady Rosetta conferred both earliness and increased tuber concentration of Cd and Zn. This is in agreement with previous work (Subramanian, 2012), which showed a major-effect QTL for tuber Zn concentration in the same location as the maturity locus on chromosome 5. In that study, the author suggested that increased tuber yield of the late-maturing genotypes may reduce tuber Zn concentration through dilution.

Mechanistic evidence for maturity-related control of Cd uptake in the cross

Previous studies suggested that both tuber Cd and Zn concentration were influenced by maturity (Chapters 3 and 4). In potato, it has been shown that Cd is taken up by the main root and translocated to the canopy (Reid *et al.*, 2003) where it is subsequently sequestered by the leaves, later being remobilised to the tuber via the phloem (Dunbar *et al.* 2003; Chapters 3 and 4). A time-course study on the uptake and partitioning of Cd and Zn (Chapter 3) showed that differences in tuber Cd concentration between the two cultivars are mainly driven by a three-step process: (1) total Cd uptake, (2) root to- shoot translocation and (3) partitioning of Cd between shoot and tuber. Lady Rosetta has greater Cd uptake, greater root-to-shoot translocation and biomass-driven partitioning between shoot and tuber. Further investigation using reciprocal grafted plants derived from Cara and Lady Rosetta, and selected 16 F₁ genotypes, a subset of this mapping population, clearly suggested that biomass-driven Cd partitioning between shoot and tuber was the major factor in determining differences in tuber Cd concentration (Chapter 4). On the other hand, Zn partitioning between shoot and tuber was the main cause of differences in tuber Zn concentration (Chapters 3 and 4).

Shoot-to-tuber translocation was considerably less efficient, with a significant quantity of Cd remaining in the shoot at harvest. Overall, shoot Cd concentrations were greater in Lady Rosetta than Cara, and this seemed to be related to shoot biomass, i.e. larger shoot biomass in Cara led to less shoot Cd concentration relative to Lady Rosetta, a phenomenon which is referred to as a “dilution” effect”. Patterns of tuber and shoot Cd concentration over time were similar for each cultivar, implying that tuber Cd concentration may be regulated by shoot Cd concentration. These results are consistent with those of Jönsson and Asp (2011), who found that cultivars with more tuber Cd also had greater leaf Cd concentrations.

Thus, Cara exhibited significantly less shoot-to-tuber translocation than Lady Rosetta, where greater remobilisation and more efficient tuber loading were observed due to less shoot biomass/greater shoot Cd concentration. Maturity is a major determinant (although not the only one) of canopy biomass, so it is not surprising, given these results, to find an allele associated with earliness in Lady Rosetta which is also associated with reduced Cd concentration. Whilst it is possible that this earliness allele is simply linked to an independent gene driving less accumulation, a pleiotropic effect of maturity (via the effect of shoot biomass) seems to be a more logical explanation, given the physiological evidence. Similar arguments can be made for Zn, based on both tuber yield and foliar biomass differences related to maturity. Jönsson and Asp (2011) also reported that leaf Cd exhibited significant positive correlation with tuber Cd concentration in potato.

Phenotypic analysis and QTL mapping in other species have also highlighted associations between the concentration of Cd and/or Zn in edible parts and maturity-related biomass traits (Table 5.3, Figure 5.5). For instance, time to anthesis, shoot biomass and grain Zn concentration were influenced by the same major-effect QTL in barley (Hussain *et al.*, 2016). According to the authors, genotypes with shorter time to anthesis had decreased biomass accumulation but greater grain Zn concentration. In rice (Ishikawa *et al.*, 2005), biomass and days to heading were negatively correlated with grain-Cd concentration. In wheat, a QTL was identified on chromosome 2B for grain Cd concentration, which was co-localised with a QTL for plant height (AbuHammad *et al.*, 2016), suggesting co-ordinated control.

Non-maturity-related control of Cd and Zn uptake and distribution

Four and three additional smaller-effect QTLs were discovered for Zn and Cd concentration, respectively, none of which coincided with maturity-related QTLs; indeed, some were only discovered after maturity correction (Table 5.5). Interestingly, two of the QTLs for Cd concentration, on chromosomes 3 and 6, coincided with QTLs for Zn. On chromosome 3, overlapping QTLs explaining up to 9% and 5% of the phenotypic variation were detected for Cd and Zn concentration, respectively. The presence of a double-simplex marker resulted in an increase in the accumulation of both transitional elements. On chromosome 6, coincident QTLs for both elements, explaining up to 12.5% and 7% of the phenotypic variation, were observed for tuber Cd and Zn concentration, respectively. In this case, an associated simplex allele from Lady Rosetta, the parent with the greater Cd- and Zn-accumulating phenotype, resulted in a decrease in the tuber concentration of both elements. Two independent QTLs for Zn accumulation (on chromosomes 1 and 6) and one for Cd accumulation (on chromosome 7) were also discovered. The existence of apparently maturity-independent QTLs for tuber Cd and Zn accumulation indicates that mechanisms not related to maturity are also acting to govern the traits. This fits quite well with the observations made (Chapters 3 and 4); in addition to differences due to foliar biomass-driven partitioning, Lady Rosetta also took up more Cd than Cara. The results also suggested that the uptake difference between Cara and Lady Rosetta is set at the root level, as negligible amounts of soil Cd are transferred from stolon roots or directly to the tuber in potato (Reid *et al.*, 2003). If this is the case, then it is probable that maturity- independent QTLs determine traits involved in root uptake (and possibly root-to-shoot transport) of Cd (and Zn).

Given the coincidence and shared direction of effect of some of the QTLs, some of these mechanisms control both Cd and Zn uptake and distribution. Conversely, existence of independent QTLs for the accumulation of both elements indicates that the control of their fate in the plant is not completely shared. Evidence for a combination of overlapping and independent control of the uptake/transport of Cd and Zn, and additionally Fe, was recently reviewed in rice by Yoneyama *et al.* (2015). The authors outline that the uptake of these elements at root-surface membranes is driven by specific transporters some of which have a shared affinity for all three elements, eg OsIRT1 (rice iron-regulated

transporter1). Conversely, other transporters are more specific for Zn, eg OsZIP1 ((zinc-regulated transporter/iron-regulated transporter-like protein) whilst others are specific to Cd, eg OsNRAMP5 (rice natural resistance-associated macrophage protein5). The subsequent fate of both elements in rice plants is also determined by a mixture of shared and individual processes and proteins, many of which have analogues in dicots such as *Arabidopsis* (Yoneyama *et al.*, 2015). It seems reasonable to assume that maturity independent QTLs for Cd and Zn concentration identified in this cross may represent allelic variants of potato orthologues of genes encoding such proteins, or trans-acting elements controlling their expression.

5.1.1. Selecting for potato genotypes accumulating increased Zn and reduced Cd concentrations in tubers

As previously mentioned, the phenotypic and genetic correlations may be significant in the context of breeding efforts focused on biofortification of essential micronutrients such as Zn. Phenotypic selection for large Zn individuals accumulating increased Zn concentrations may predispose the resulting varieties to accumulate undesirable concentrations of Cd on soils exhibiting greater than normal Cd concentrations. Similar observations were reported in other crops, namely barley (Chen *et al.*, 2007; Wu *et al.*, 2015), rice (Zhang *et al.*, 2011) and soybean (Sugiyama *et al.*, 2011). However, the association between the two elements is not complete, and, even in the mapping population used in this study, it is possible to identify individual genotypes which accumulate greater concentrations of tuber Zn but which have tuber Cd concentrations which are acceptable by current international standards.

Figure 5.6 shows the relationship between Cd and Zn values in the population, based on the BLUE analysis. The x-axis represents the population grand mean for Cd, and (by chance) approximates the acceptable level of Cd in potato tubers (0.1 mg kg⁻¹ fresh weight) set in most jurisdictions. The five individuals with the largest Zn concentrations but falling below the x-axis are highlighted. Of these five, four have a maturity score of 8-9, indicating a very early phenotype, whilst one exhibits a score of 3. Interestingly, all five, including the latter, possessed the simplex allele derived from Lady Rosetta and associated with earliness. These observations are in line with the fact that the correlation

with maturity is greater for tuber Zn than for tuber Cd, and may even indicate that different maturity-related processes are important for the two traits. When the five individuals were examined for their marker genotype at the small-effect QTLs for tuber Cd-accumulation independent of Zn (on chromosomes 3 and 7), they did not consistently exhibit the allelic configuration predicted to lower Cd concentration. This suggests that, when selecting for acceptable Cd levels, earliness (or possession of the earliness-related QTL) is a better predictor than the possession of the Cd-lowering configuration at these small-effect loci.

Thus, in this cross at least, the prospect of using MAS for the development of increased-Zn/ reduced Cd varieties is not promising. However, the study clearly shows the existence of maturity-independent QTLs for tuber Cd concentration, probably governing processes such as root uptake. Cd accumulation is likely to be under complex genetic control, and, in this single cross, we are unlikely to have discovered either all of the loci involved in the process, or the full extent of allelic variation at the loci we did discover. Significantly, loci affecting Cd but not Zn accumulations exist which means that selection is at least possible in theory. A breeding programme seeking to specifically develop “Cd-safe”, high-Zn potato varieties would be advised to screen a greater diversity of material to identify more tuber-Cd-specific loci, which could be accumulated via an MAS programme. Accumulation of a sufficient number of these loci could counteract the effect of biomass-partitioning-driven tuber Cd-accumulation by reducing the overall Cd available in the plant in the first instance.

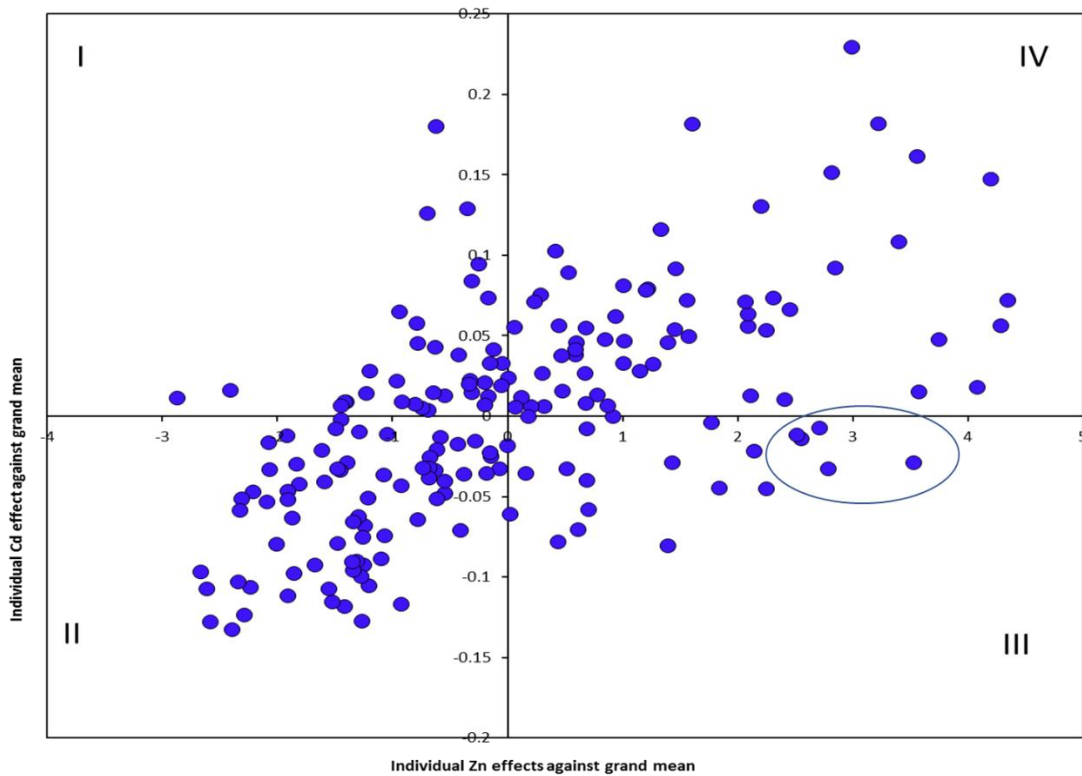


Figure 5.6 Correlation between tuber Cd and Zn concentrations in 188 F₁ genotypes derived from Cara and Lady Rosetta. The x-axis represents the mean values of Zn concentration measured over two years and the points are individual effects against the grand mean (10.212 mg kg⁻¹ dry weight). Similarly, the y-axis represents the mean values of Cd concentration of the mapping population measured over two years and the points are individual effects against the grand mean (0.451 mg kg⁻¹ dry weight). The mean value of Cd concentration of the mapping population is close to the maximum limit of Cd allowed in potato (0.1 mg kg⁻¹ fresh weight), based on an assumption of approximately 25% total dry matter content on average in potato. R² is the coefficient of determination of the two variables (Cd and Zn). The five genotypes exhibiting the largest Zn levels but falling below the maximum acceptable level of tuber-Cd concentration, as mentioned in the text, are highlighted (black oval). Quadrants indicate more Cd and less Zn (I), less Cd and less Zn (II), less Cd and more Zn (III) and more Cd and more Zn (IV) when compared against the respective grand mean of the two transitional elements.

Chapter 6: General Discussion

6.1. Introduction

This PhD project was carried out to provide some insights into the major physiological and genetic mechanisms involved in determining inter-genotype differences in tuber Cd and Zn concentrations, with potential applications to food safety and biofortification perspectives. In this chapter, the main findings, implications for potato breeding, research limitations and future research directions will be addressed.

Geogenic origin of Cd is the main driver for this specific project (Fay *et al.*, 2007). Furthermore, agricultural practices such as sewage sludge, manure, and increased application of phosphate fertiliser contribute to increasing the level of Cd in agricultural soils (Clemens, 2006; Roberts, 2014). The increased Cd concentrations in agricultural soil pose serious health risks and require regulatory measures to reduce Cd levels in the food chain (Grant *et al.*, 2008). On the other hand, deficiencies of essential elements, including Zn, are one of the main health risk factors in developing countries, where the problem is prevalent (Erenoglu *et al.*, 2011; Sperotto, 2013; White *et al.*, 2017). Therefore, breeding programmes should take into account the nutritional value of essential trace elements in the crop while reducing the bioavailability of Cd, particularly if reduced Cd accumulation was included as a selection criterion (Grant *et al.*, 2008). On the other hand, it is also important to note that breeding for a high-Zn phenotype may also accumulate greater tuber Cd concentrations in situations where Cd is problematic. Given that potato is an important crop for food security and nutritional values, reducing Cd concentrations while increasing Zn concentrations in the tubers is a valid objective in breeding programmes, particularly those focused on developing countries. To develop genotypes and/or agronomic management practices that result in reduced Cd and increased Zn concentrations in potato tubers, understanding the genetic and physiological bases and commonalities in the accumulation of the two elements is indispensable.

6.2. The two cultivars differed in total Cd uptake but not Zn uptake

As has been previously demonstrated, the route of movement of Cd and Zn to the tuber follows the xylem and phloem pathways (Reid *et al.*, 2003). Both elements are taken up by the basal roots, translocated to the shoot via the xylem and redistributed to the tuber via the phloem (Kratzke & Palta, 1985, 1986; Dunbar *et al.*, 2003; Reid *et al.*, 2003).

During the processes of “import and export”, factors associated with uptake, transport and distribution can determine the final tuber Cd and Zn concentrations (Kidd *et al.*, 2009; Sheoran *et al.*, 2016).

Plant accumulation of transitional elements, including Cd and Zn, depends on the soil element concentrations, soil and plant factors associated with element availability in the rhizosphere, and the genotypic ability to absorb the elements (Kidd *et al.*, 2009; Sheoran *et al.*, 2016). In Chapter 3, the two cultivars, Cara and Lady Rosetta, were evaluated for their Cd and Zn uptake abilities as measured by plant Cd and Zn uptake.

The two cultivars differed markedly with respect to Cd uptake, with Lady Rosetta taking up 20% more Cd than did Cara. Similarly, the differences in total Cd uptake between the two cultivars were confirmed, using reciprocal grafted plants (Chapter 4). Furthermore, the study provided some new insights, that the differences in Cd uptake between the two cultivars were driven by root-related traits. The observation of an uptake difference between cultivars is novel; a previous study by Dunbar *et al.* (2003) did not observe differences in the Cd uptake between two Australian potato cultivars with very different final tuber Cd concentrations (‘Wilwash’ and ‘Kennebec’). Dunbar *et al.* (2003) grew plants in sand supplemented by 1 L/day 10 nM Cd solution, which represented far lower Cd levels than were used in the current study ($\sim 1 \text{ mg kg}^{-1}$ soil). In addition, the total plant Cd content at final harvest in the Australian study was 48 μg , which was considerably less than in this study ($\sim 362 \mu\text{g}$ for Cara and $\sim 448 \mu\text{g}$ for Lady Rosetta). These differences between the studies in terms of the concentration of Cd in the source medium (leading to potentially greater uptake levels in the current study) may have masked any potential uptake differences in the Australian study.

In wheat, studies by Hart *et al.* (1998) and Harris and Taylor (2001, 2004, 2013) reported that differences in grain Cd concentration were not associated with differences in Cd uptake, but differed in partitioning of Cd between plant organs. However, recent studies (Arduini *et al.*, 2014; Kubo *et al.*, 2016; Perrier *et al.*, 2016) have reported that differences in Cd uptake between wheat cultivars contributed to inter-cultivar differences in grain Cd concentration. Furthermore, Arduini *et al.* (2014) found that differences in Cd uptake by durum wheat cultivars were highly dependent on the amount of Cd in the soil. At low soil

Cd, between-cultivar differences were not observed, whereas clear differences were observed as the Cd supply in the soil increased. At low soil Cd concentration, it was difficult to exploit genotypic potential for altered total Cd uptake.

6.3. Root-to-shoot translocation was effective for both Cd and Zn

Once Cd or Zn enters the root cell, it is either retained in the root or translocated to the shoot. Previous studies in potato (Dunbar *et al.*, 2003) and other solanaceous species (Mori *et al.*, 2009) reported that root-to-shoot translocation is an important factor in determining differences in Cd distribution. However, research in this study (Chapter 3) showed that root-to-shoot translocation was effective for both Cd and Zn, with greater translocation efficiency exhibited by Lady Rosetta than by Cara.

Unlike cereal or legume crops, the relative contribution of root dry weight to the total plant dry weight accumulated in potato is insignificant, usually less than 5% (Soratto *et al.*, 2015; Soratto & Fernandes, 2016; Kromann *et al.*, 2017). As Cd or Zn content in a given organ is the resultant of “concentration” by “dry weight” of that organ, root Cd or Zn concentration would need to be exceptionally large to accumulate markedly greater Cd or Zn content. In fact, the root Cd or Zn concentration under low to medium soil Cd ($\sim 1 \text{ mg kg}^{-1}$) or Zn concentrations ($\sim 73 \text{ mg kg}^{-1}$ soil) was similar to or slightly larger than that in the shoot in this study. Taking these data into account, root-to-shoot translocation could not be a major factor in determining inter-cultivar differences in tuber Zn concentrations, where the level of soil Zn is not greater than 73 mg kg^{-1} Zn soil. Similarly, genetic intervention, focused on reducing the Cd concentration by sequestering it in the root, may not be promising in situations with low soil Cd concentration ($\sim 1 \text{ mg kg}^{-1}$ soil), as the contribution of root Cd content to the total plant Cd content is less than 5%. Conversely, the role of roots in Cd sequestration was substantial where soil Cd concentration was large. At these high soil Cd concentrations ($\sim 5 \text{ mg kg}^{-1}$ soil), the amount of Cd accumulated in the root was greater than the amount of Cd accumulated in the tuber in all grafted combinations (Chapter 4). Overall, in terms of real-world agronomic significance for Cd accumulation in potato, it is possible to conclude that the role of roots is more important in terms of Cd uptake than sequestration.

6.4.Partitioning between shoot and tuber was the major factor determining differences in tuber Cd and Zn concentration, but different mechanisms prevail

6.4.1. Shoot biomass-associated “dilution effect” plays a marked role in determining differences in tuber Cd concentration

Results from Chapter 3 revealed that differences in total uptake, root-to-shoot translocation and partitioning between shoot and tuber contributed to differences in tuber Cd concentration. The results also reflected the importance of shoot biomass in regulating shoot and tuber Cd concentration, mainly via growth dilution. In the subsequent study (Chapter 4), using reciprocal grafted plants and selected F₁ genotypes derived from the two cultivars, the contribution of these variables for differences in tuber Cd concentration was evaluated. According to this study, Cd partitioning between shoot and tuber was a major cause for differences in tuber Cd concentration. Despite greater plant Cd uptake by a Cara scion grafted onto a Lady Rosetta rootstock, the contribution to differences in tuber Cd concentration was minor, as compared to self-grafted Cara or non-grafted Cara. Similarly, selected F₁ genotypes clearly illustrated that genotypic variability in shoot biomass affected shoot Cd concentration, and the relationship between shoot and tuber Cd concentrations (Chapter 4). Consistent with results from Chapters 3 and 4, phenotypic analysis of 188 F₁ genotypes derived from the two cultivars showed a strong negative correlation between tuber Cd concentration and maturity. QTL analysis also revealed that a major-effect genetic component controlling tuber Cd concentration coincides with one controlling maturity (Chapter 5). The importance of shoot biomass in regulating grain Cd concentration has been reported from other crops, such as wheat (Arduini *et al.*, 2014; Perrier *et al.*, 2016) and rice (He *et al.*, 2006).

Another interesting finding from this study (Chapter 3) was that, regardless of cultivars, remobilisation of stored Cd from vegetative organs was the main source of Cd to the developing tuber. As tubers are non-transpiring organs and do not require Cd for any biological processes, active transport of Cd to the tuber is unlikely. Instead, Cd is transported to the shoot via the xylem during the vegetative stage, as a direct consequence of greater transpiration. The shoot-stored Cd is then remobilised to the tuber via the

phloem, along with other photoassimilates (Reid *et al.*, 2003). Given that remobilisation is the main source of tuber Cd, it is not surprising that proportionality of Cd concentration between the shoot and tubers is a driving factor for the differences in Cd translocation to the tuber. The results agreed with those of previous reports (Hart *et al.*, 1998; Tanaka *et al.*, 2007; Kashiwagi *et al.*, 2009; Rodda *et al.*, 2011), that remobilisation of Cd from vegetative organs is the main source of phloem Cd, which is translocated to the edible sink, such as tubers.

6.4.2. Competition between organs for Zn is the most likely reason for differences in tuber Zn concentration

Unlike Cd, the two cultivars showed no significant differences in either total Zn uptake or root-to-shoot translocation. Instead, Zn partitioning between stem and tuber was the main cause for differences in tuber Zn concentration between the two cultivars (Chapter 3). Examination of the partition quotient (PQ), regardless of plant size, showed that the stem had greater PQ values than did the other organs for both cultivars. Furthermore, the two cultivars had similar stem PQ values, demonstrating that the greater tuber Zn content of Cara over Lady Rosetta was not associated with preferential distribution of Zn from the stem, but rather by differences in stem dry weight (Chapter 3).

In Chapter 4, results from reciprocal grafted plants confirmed that Zn partitioning between the stems and tubers was the main cause of differences in tuber Zn concentration between the two cultivars. Further correlation analysis of biomass-related traits with Zn concentration in different organs showed that leaf biomass did not show any association with leaf Zn or tuber Zn concentration (Chapter 4). Instead, the trends showed that tubers and the shoot competed for Zn. These results agreed with previous reports (Almekinders & Struik, 1996) that shoot and tuber growth are generally considered as competing processes in the potato plant. Consistent with the findings in Chapters 3 and 4, phenotypic evaluation of the 188 F₁ genotypes, from a cross between these two cultivars, showed that tuber Zn concentration exhibited a strong negative association with maturity, with early-maturing genotypes accumulating more tuber Zn than did late-maturing genotypes.

QTL analysis also revealed that a major-effect QTL for tuber Zn concentration co-located with a maturity QTL (Chapter 5). Based on the results that competition occurs between the shoot and tubers for Zn (Chapter 4), it is possible to conclude that late-maturing genotypes demanded more Zn for shoot growth, at the expense of the tubers, compared to early-maturing genotypes. Hence, competition for Zn between shoots and tubers, rather than growth dilution, was the main cause for differences in tuber Zn concentration between the two cultivars.

6.5. Comparison of Cd and Zn

For both transitional elements, partitioning between shoot and tuber is an important driver for differences in accumulation in the tubers. Furthermore, the processes of partitioning between the shoot and tubers of both transitional elements were heavily influenced by biomass (growth habit differences) but for different reasons. For Cd, the main effect of biomass on differences in tuber Cd concentration was driven by a growth-associated dilution effect. As Zn is an essential element for plant growth and development, competition between organs for Zn is expected, with late-maturing genotypes demanding more Zn for shoot growth at the expense of the tuber (Trudgill & Thompson, 1987; Pearson & Rengel, 1995; Kooman & Rabbinge, 1996; Almekinders & Struik, 1996; Yamaji & Ma, 2014).

Overall, the genetic and physiological mechanisms of Zn and Cd accumulation are different in a number of respects. Firstly, the two cultivars did not show differences in Zn uptake, contrary to Cd uptake, where Lady Rosetta had significantly greater uptake than did Cara. Secondly, as Zn is an essential element for plant growth and development, Zn distribution is not simply driven by transpiration via the xylem. Instead, Zn is highly regulated and preferentially distributed between organs, depending on the relative strengths of the different sinks (Almekinders & Struik, 1996). As leaves and tubers require Zn for physiological processes to operate properly, it is not surprising that strong competition is observed between the two organs (Almekinders & Struik, 1996). Another interesting observation was that Zn concentration in organs, including the tubers, was less influenced by shoot biomass than was Cd concentration. Furthermore, considerably more (> 70%) of the whole-plant Zn was translocated to the tubers at final harvest compared to

that of Cd, where a maximum value of 50% was observed. Therefore, it is important to note that the fate of Cd and Zn inside the plants is not completely shared.

6.6. Potato breeding implications

6.6.1. Maturity as a phenotypic marker

Differences in biomass accumulation among cultivars are an important but underappreciated factor in most transitional element accumulation studies. It is well established that Cd is mainly transported to the above-ground plant parts following the transpiration stream. The fate of Cd in the above-ground plant parts is either storage in the shoot or translocation to the tuber and/or to the root. Before tuber initiation, the shoot is the only destination for Cd. When the plants start tuber initiation, shoot-stored Cd is expected to be remobilized and translocated to the tuber, along with photoassimilates.

Differences in shoot biomass production can also affect the shoot Cd concentration through a dilution effect. Shoot-to-tuber Cd translocation was not particularly efficient, with a significant amount of Cd remaining in the shoot at harvest. Nevertheless, the two cultivars exhibited significant differences in both shoot Cd concentration and leaf Cd remobilisation (with the cultivar accumulating greater concentrations of Cd in the tuber also exhibiting greater shoot Cd concentration and remobilisation), driven by growth differences associated with biomass production. Similarly, tuber Zn concentration was influenced by biomass production but for different reasons.

As mentioned above, it is possible to predict tuber Cd and Zn concentrations using maturity as phenotypic marker, with early-maturing genotypes tending to accumulate greater tuber Cd and Zn concentrations than late-maturing genotypes. Late-maturing genotypes generally have greater tuber yield than do early-maturing genotypes (Bradshaw *et al.*, 2006, 2008). Interestingly, the information generated in this study provides an insight into the development of greater yielding genotypes which accumulate reduced concentrations of tuber Cd in the same breeding programme. However, it is difficult to select for increased tuber yield and increased Zn concentration simultaneously. Other management options may be required to optimise tuber yield and enhance tuber Zn concentration.

6.6.2. Agronomic management

The information generated from both physiological and genetic analysis confirmed the importance of shoot biomass in reducing tuber Cd concentration. As shoot biomass is a means to reduce tuber Cd concentration, it is therefore important to develop agronomic interventions to increase shoot biomass without affecting important agronomic traits including tuber yield.

Nitrogen fertilizer is known to increase the biomass of plants. An increase in shoot biomass is expected to reduce Cd concentration and also the ability of the plant to translocate Cd to the edible plant parts (Sarwar *et al.*, 2010). However, studies have reported inconsistencies concerning the effect of N on Cd concentrations in edible portions of the plant. Li *et al.* (2011) suggested that increased N application increased Cd concentration in edible plant parts, including grains of wheat. Conversely, another study showed that application of nitrogen fertilizer reduced Cd concentration in wheat grains (Landberg and Greger 2003).

In potato, Jonsson and Asp (2011) reported that increased application of N fertilizer reduced tuber Cd concentration. The authors reported that increasing the amount of N fertilizer applied from 60 to 160 or 240 kg N ha⁻¹ reduced tuber Cd concentration, regardless of the cultivars being tested, the effect being explained as a “dilution effect”, caused by increased biomass. Studies have suggested that the effects of N fertilization on Cd concentration may differ with the N source and plant species, because of their different preferences for absorbing and utilizing nitrate and ammonium (Grant *et al.*, 1999; Jonsson and Asp, 2011). Studies showed that plant Cd concentration was increased when they were supplied with acidifying fertilizers such as ammonium sulphate (Grant *et al.*, 1999; Jonsson and Asp, 2011). However, the N fertilizer effect is influenced by the initial soil pH and the buffering capacity of the soil and counter-ion (e.g. Ca²⁺) applied with the N fertilizer (Grant *et al.*, 1999; Jonsson and Asp, 2011). According to Jonsson and Asp (2011), tuber Cd concentration increased more when plants were provided with N in the form of ammonium sulphate than as a neutral compound NPK fertilizer, the effects being explained as increased soil Cd availability due to low soil pH and thereby increased plant

Cd accumulation. On the other hand, a study by Cheng *et al.*, (2016) showed that increases in uptake and accumulation of Cd in nutrient solution containing ammonium was not the result of altered bioavailability of Cd or increased plant growth. Instead, Cd accumulation was influenced by complex physiological processes, including Cd uptake, transport and distribution in the plant. The authors explained that the N form may change root-related traits by explaining the important effect of ammonium for root cell division and for expression of genes involved in the transport and distribution of Cd. Due to the complex nature of soil-plant interactions and the status of the soil, it is important to evaluate the different N forms under given conditions before applying N management practices with the aim of reducing Cd in the edible plant parts.

Another interesting observation was that shoot biomass did not show any positive association with Cd uptake, as the cultivar which accumulated the smaller shoot biomass, Lady Rosetta, had greater Cd uptake than Cara. White *et al.* (2012) found that increased N application resulted in an increased Zn concentration in potato tubers. Similar results were reported in wheat (Erenoglu *et al.*, 2011), where increased N application boosted root uptake, root-to-shoot translocation and remobilisation of Zn. Therefore, N nutrition management might be an agronomic management option to help grow potatoes combining increased tuber Zn concentration with reduced tuber Cd concentration.

6.7. Limitations and future researches

Despite the two cultivars being selected for their extreme Cd concentration, they also showed extremes with respect to important traits such as maturity class and root system size. Differences in maturity and root system size affected tuber Cd and Zn concentrations in this study. Therefore, it was not possible to fully exploit maturity-independent traits related to tuber Cd and Zn concentration. Furthermore, despite the two cultivars being different with respect to Cd uptake, with the differences being driven by root-related traits, results from this work were limited to identifying the most important root-related traits which drove differences in Cd uptake between the two cultivars. Therefore, further study will be needed to identify the most important root-related traits and to incorporate these traits as a screening tool in potato breeding programme. Another interesting observation was that much more Cd was sequestered in the shoot, as compared to the tuber. Therefore,

it is important to understand which underlying mechanisms maintained the greater concentration gradients between shoot and tubers. To provide an insight about this mechanism(s), it is advisable to study the chemical forms and subcellular localizations of Cd in the shoot. Further study will be needed to answer this question. Finally, the study showed that the stem is the most important organ in determining differences in tuber Zn concentration. However, the study was limited with respect to exactly how the stem affects tuber Zn distribution, either as a site for deposition and/or a Zn-trafficking centre. Future study will be required to achieve a greater understanding of how the stem determines tuber Zn distribution.

In conclusion, the main findings of this study are:

- ✓ Differences in tuber Cd concentration are influenced by a three-step process: total plant Cd uptake, root-to-shoot Cd translocation and Cd partitioning between shoot and tuber.
- ✓ The roots are an important organ for Cd uptake but less important for determining growth, tuber Cd concentration and root Cd sequestration.
- ✓ The shoot is an important organ for growth and is a determining factor for tuber Cd concentration.
- ✓ Most of the QTLs for Cd and Zn are co-located, meaning that simultaneous selection for genotypes accumulating increased Zn but decreased Cd concentrations in the tubers in plants grown in Cd- contaminated soil, where Cd contamination of the food chain is a serious problem is not promising.
- ✓ Differences in growth habit affected both tuber Cd and Zn concentrations but for different reasons. Growth habit affected tuber Cd concentration mainly as a result of growth dilution. On the other hand, the competition for Zn between shoot and tuber at early stages of growth may be the most likely reason for the differences in tuber Zn concentrations between the two cultivars

Chapter 7: References

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Chapter 8: Appendix

Table 8.1 Analysis of variance for each component

Parameter	Source of variance	DF	SS	F Value	Pr > F
Total DW per plant	time	6	282555	33.32	<.0001
	cultivar	1	10242.3	7.25	0.02
	time*cultivar	6	19454.3	2.29	0.1
Stem Cd concentration	time	7	92.15	12.53	<.0001
	cultivar	1	231.95	220.84	<.0001
	time*cultivar	7	93.48	12.71	<.0001
Stem Cd content	time	7	46415	45.5	<.0001
	cultivar	1	15.82	0.11	0.75
	time*cultivar	7	2415.34	2.37	0.07
Stem DW	time	7	808.39	42.77	<.0001
	cultivar	1	492.84	182.51	<.0001
	time*cultivar	7	168.66	8.92	0
tuber Cd concentration	time	7	0.1	5.77	0
	cultivar	1	1.34	522.93	<.0001
	time*cultivar	7	0.08	4.57	0.01
Tuber Cd content	time	7	112908	34.34	<.0001
	cultivar	1	56331.2	119.94	<.0001
	time*cultivar	7	3884.97	1.18	0.37
Tuber DW	time	7	367748	45.1	<.0001
	cultivar	1	7921.94	6.8	0.02
	time*cultivar	7	30183	3.7	0.02
Root Cd concentration	time	8	416.46	32.25	<.0001
	cultivar	1	42.6	26.39	<.0001
	time*cultivar	8	29.9	2.32	0.07
Root Cd Content	time	8	2550.18	35.82	<.0001
	cultivar	1	4987.01	560.42	<.0001
	time*cultivar	8	740.92	10.41	<.0001
Root DW	time	8	21.94	72.64	<.0001
	cultivar	1	42.95	1137.96	<.0001
	time*cultivar	8	6.53	21.63	<.0001
Leaf Cd concentration	time	7	142.04	9.58	<.0001
	cultivar	1	65.76	35.47	<.0001
	time*cultivar	7	77.95	5.26	0
Leaves Cd content	time	7	92802.9	17.64	<.0001
	cultivar	1	2074.77	3.16	0.09

Table 8.1 Analysis of variance for each component

Parameter	Source of variance	DF	SS	F Value	Pr > F
	time*cultivar	7	7414.36	1.41	0.26
Leaf DW	time	8	1316.22	37.89	<.0001
	cultivar	1	309.75	71.33	<.0001
	time*cultivar	8	134.62	3.88	0.01
Total Cd per plant	time	5	103459	10.66	0
	cultivar	1	15307.1	9.46	0.01
	time*cultivar	5	19567.6	2.02	0.14
Cd root-to-shoot translocation (%)	time	8	1973.69	63.22	<.0001
	cultivar	1	424.481	108.77	<.0001
	time*cultivar	8	76.4155	2.45	0.0547

For total DW per plant and total Cd per plant, the analysis of variance was performed from 57 DAP to final harvest.

Table 8.2 Total dry weight accumulation per plant over time with respective to the cultivar

Days after planting	Cara	Lady Rosetta
30	11 ^e	8 ^e
45	37 ^{de}	29 ^e
57	116 ^{cd}	129 ^d
73	154 ^{bc}	196 ^{cd}
84	232 ^b	223 ^{bc}
98	333 ^a	268 ^{abc}
110	347 ^a	286 ^{abc}
125	366 ^a	298 ^{ab}
150	402 ^a	328 ^a

Table 8.3 Dry weight accumulation between the two cultivars in different organs over time (only for those significant cultivar*time).

Tuber DW					
DAP	DF	SS	MS	F Value	Pr > F
45	1	35.58	35.58	0.04	0.84
57	1	1237.26	1237	1.41	0.26
73	1	6076.35	6076	6.91	0.02
84	1	218.29	218	0.25	0.63
98	1	2496.96	2496	2.84	0.11
110	1	1393.16	1393	1.58	0.23
125	1	10316	10316	11.73	0
150	1	16420	16420	18.68	0
Root DW					
30	1	0.05	0.05	1.34	0.26
45	1	2.72	2.72	72.07	<.0001
57	1	12.41	12.41	328.86	<.0001
73	1	6.59	6.59	174.7	<.0001
84	1	5.51	5.51	145.99	<.0001
98	1	5.04	5.04	133.57	<.0001
110	1	8.03	8.03	212.67	<.0001
125	1	4.82	4.82	127.81	<.0001
150	1	4.3	4.3	113.95	<.0001
Stem DW					
30	1	0.44827	0.4483	0.17	0.6899
45	1	27.478	27.48	10.18	0.0066
57	1	131.977	131.977	48.87	<.0001
73	1	36.992	36.9922	13.663	0.02614
84	1	154.432	154.432	57.19	<.0001
98	1	154.94	154.94	57.38	<.0001
110	1	247.042	247.042	91.48	<.0001
125	1	155.09	155.09	67.53	0.001
150	1	78.844	78.8438	29.2	<.0001
Leaf DW					
30	1	6.9696	6.969	1.6	0.2259
45	1	16.52	16.524	3.81	0.0714
57	1	0.207	0.207	0.05	0.8303
73	1	11.179	11.179	2.57	0.1309
84	1	112.753	112.753	25.97	0.0002
98	1	86.118	86.118	19.83	0.0005
110	1	164.431	164.431	37.87	<.0001
125	1	73.71	73.71	16.97	0.001
150	1	15.844	15.843	9.65	0.0368

Table 8.4. Analysis of variance for tubers Cd concentration over time

Source	DF	Cara				Lady Rosetta			
		SS	MS	F Value	Pr > F	SS	MS	F Value	Pr > F
Time	7	0.0356	0.0051	2.52	0.071	0.1469	0.1469	5.91	0.0024
Rep	2	0.0004	0.0002	0.11	0.8965	0.017	0.017	2.39	0.1278

Table 8.5 Cd concentration between the two cultivars in different organs over time (only for those significant cultivar*time)

Tuber Cd concentration					
DAP	DF	SS	MS	F Value	Pr > F
45	1	0.061	0.061	16.96	0.0009
57	1	0.0641	0.0641	17.81	0.0007
73	1	0.1014	0.1014	28.18	<.0001
84	1	0.3504	0.3504	97.4	<.0001
98	1	0.2321	0.2321	64.5	<.0001
110	1	0.1667	0.1667	46.32	<.0001
125	1	0.2054	0.2054	57.08	<.0001
150	1	0.198	0.198	55.04	<.0001
Stem Cd concentration					
30	1	6.0803	6.0803	5.79	0.0286
45	1	13.4102	13.4102	12.77	0.0025
57	1	3.84	3.84	3.66	0.0739
73	1	0.2204	0.2204	0.21	0.653
84	1	73.7803	73.7803	70.25	<.0001
98	1	51.4508	51.4508	48.99	<.0001
110	1	99.6338	99.6338	94.86	<.0001
150	1	77.0058	77.0058	73.32	<.0001
Leaves Cd concentration					
30	1	2.4448	2.4448	1.32	0.2677
45	1	7.4148	7.4148	4	0.0628
57	1	0.0294	0.0294	0.02	0.9014
73	1	0.7704	0.7704	0.42	0.5283
84	1	1.3728	1.3728	0.74	0.4022
98	1	23.562	23.562	12.71	0.0026
110	1	45.0241	45.0241	24.29	0.0002
150	1	52.7474	52.7474	28.45	<.0001

Table 8.6 Analysis of Variance for each component of Zn concentration and content

Parameters	Source of variance	DF	Sum square	Pr > F
Leaf Zn concentration	cultivar	1	431	0.00
	time	8	4561	<.0001
	cultivar*time	8	498	0.06
Root Zn concentration	cultivar	1	94	0.22
	time	8	2495	0.00
	cultivar*time	8	426	0.52
Stem Zn concentration	cultivar	1	8039	<.0001
	time	8	9026	0.01
	cultivar*time	8	3415	0.21
Tuber Zn concentration	cultivar	1	62	<.0001
	time	7	558	<.0001
	cultivar*time	7	54	0.01
Leaf content	cultivar	1	15821	0.16
	time	8	1708688	<.0001
	cultivar*time	8	74629	0.32
Stem content	cultivar	1	1552925	<.0001
	time	8	4843898	<.0001
	cultivar*time	8	258079	0.54
Root content	cultivar	1	94051	<.0001
	time	8	47585	<.0001
	cultivar*time	8	14867	0.01
Tuber content	cultivar	1	1909240	0.00
	time	7	41393870	<.0001
	cultivar*time	7	725402	0.44
total content	cultivar	1	44406	0.59
	time	8	96773014	<.0001
	cultivar*time	8	635724	0.82

For total DW per plant and total Zn content per plant, the analysis of variance was performed from 57 DAP to final harvest.

Table 8.7 combined analysis of variance for tuber Cd and Zn concentrations

Tuber Cd concentration					
Source	DF	MS	Expected Mean Square	F Value	Pr > F
G	187	0.047	$\text{Var}(\text{Residual}) + 2.9999 \text{ Var}(\text{geno}*\text{YR}) + \text{Q}(\text{geno})$	4.37	<.0001
G*YR	187	0.011	$\text{Var}(\text{Residual}) + 2.9999 \text{ Var}(\text{geno}*\text{YR})$	2.28	<.0001
YR	1	12.84	$\text{Var}(\text{Residual}) + 188 \text{ Var}(\text{rep}(\text{YR})) + 563.99 \text{ Var}(\text{YR}) + 3 \text{ Var}(\text{geno}*\text{YR})$	48.16	0.0019
rep(YR)	4	0.26	$\text{Var}(\text{Residual}) + 187.67 \text{ Var}(\text{rep}(\text{YR}))$	55.24	<.0001
Residual	748	0.005	$\text{Var}(\text{Residual})$.	.
Tuber Zn concentration					
G	187	24.17	$\text{Var}(\text{Residual}) + 2.9999 \text{ Var}(\text{geno}*\text{YR}) + \text{Q}(\text{geno})$	5.22	<.0001
G*YR	187	4.63	$\text{Var}(\text{Residual}) + 2.9999 \text{ Var}(\text{geno}*\text{YR})$	2.27	<.0001
YR	1	380.17	$\text{Var}(\text{Residual}) + 188 \text{ Var}(\text{rep}(\text{YR})) + 563.99 \text{ Var}(\text{YR}) + 3 \text{ Var}(\text{geno}*\text{YR})$	20.89	0.0049
rep(YR)	4	15.59	$\text{Var}(\text{Residual}) + 187.67 \text{ Var}(\text{rep}(\text{YR}))$	7.63	<.0001
Residual	748	2.043	$\text{Var}(\text{Residual})$.	.

G, genotype; YR, year; rep, replication; DF, degree of freedom; SS, sum of squares; MS, mean sum of square.

Table 8.8 Summary of markers by parent and marker classes

Chrom.	Simplex ^a		Duplex ^b		Double simplex ^c	Others SNPs ^d	Sum of (^a), (^b) and (^c)	Subtotal	% simplex	% of (^a), (^b) and (^c) compared to subtotal
	Cara	LR	Cara	LR						
1	88	50	17	21	58	124	234	358	38.55	65
2	75	40	6	16	45	129	182	311	36.98	59
3	51	84	15	9	85	108	244	352	38.35	69
4	131	40	11	9	57	142	248	390	43.85	64
5	59	50	6	13	34	59	162	221	49.32	73
6	55	77	6	22	49	146	209	355	37.18	59
7	94	55	16	4	87	133	256	389	38.30	66
8	63	36	9	10	79	122	197	319	31.03	62
9	79	51	11	17	47	118	205	323	40.25	63
10	59	29	13	8	28	101	137	238	36.97	58
11	103	75	3	4	50	78	235	313	56.87	75
12	38	46	2	20	23	54	129	183	45.90	70
Total	895	633	115	153	642	1314	2438	3752	41.41	65

^a Simplex marker segregate at 1:1 (AAAA x AAAB).

^b Duplex markers segregate at 1:4:1(AAAA x AABB).

^c Double simplex markers segregate at 1:1(AAAB x AAAB).

^d Other markers include markers which do not describe in

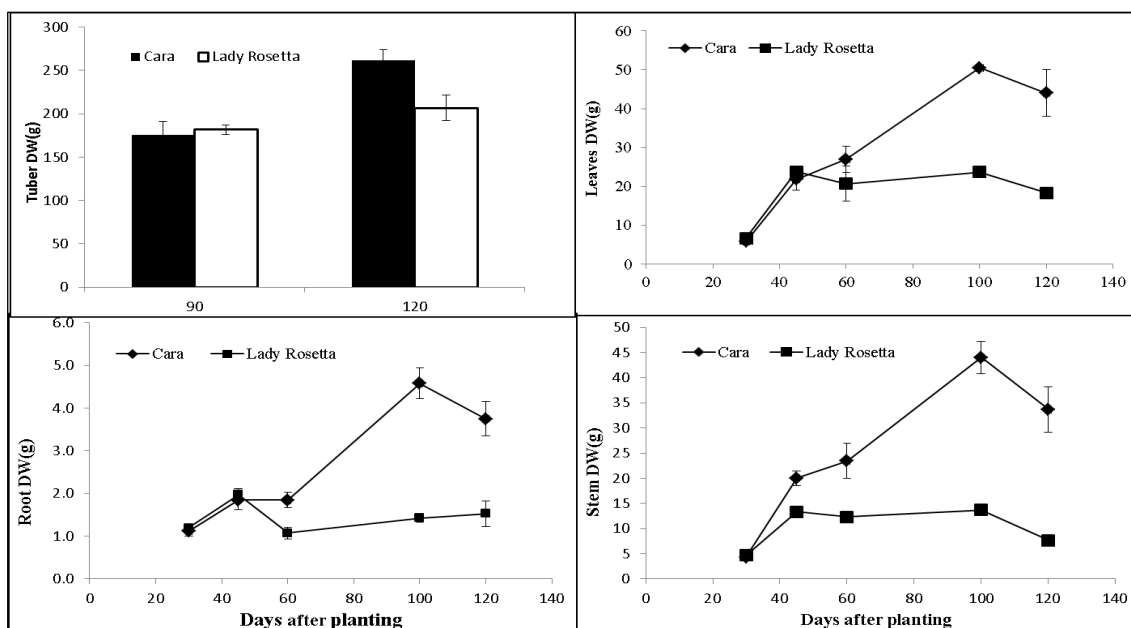


Figure 8.1 DW accumulation in different organs of Cara and Lady Rosetta over time. Data points represent the mean \pm standard error of the mean (n=3), 2015 data.

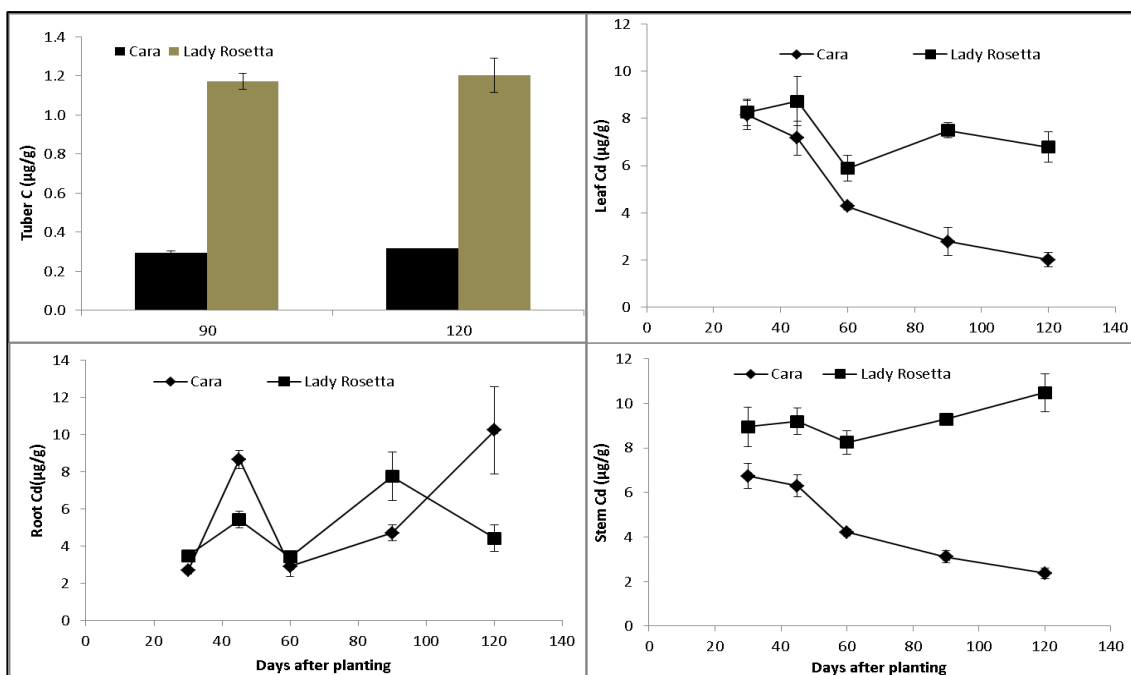


Figure 8.2 Cd concentrations of different organs of Cara and Lady Rosetta over time. Data points represent the mean \pm standard error of the mean (n=3), 2015 data.

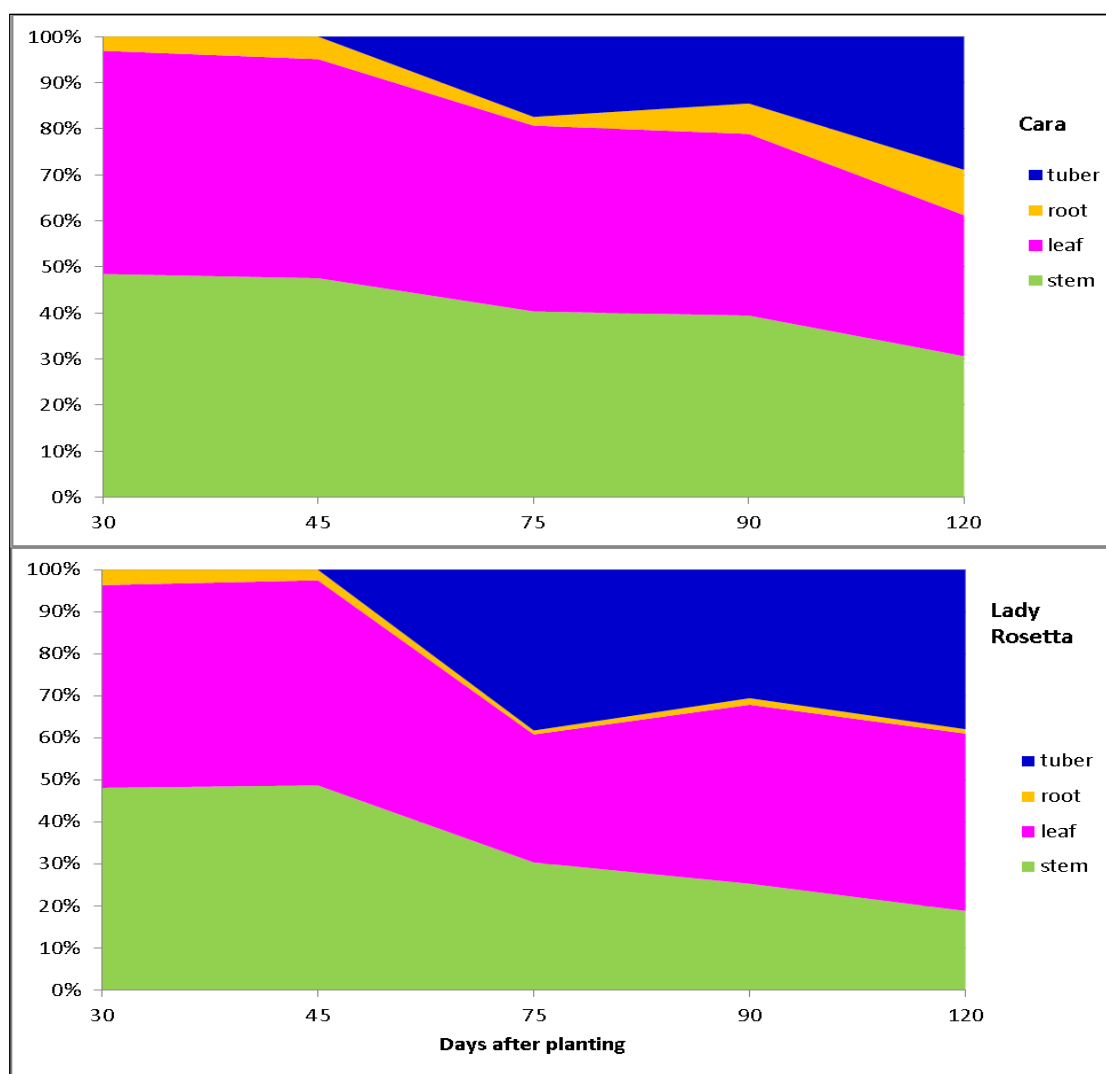


Figure 8.3 Distribution of Cd in all organs of potato plants over the growth period.

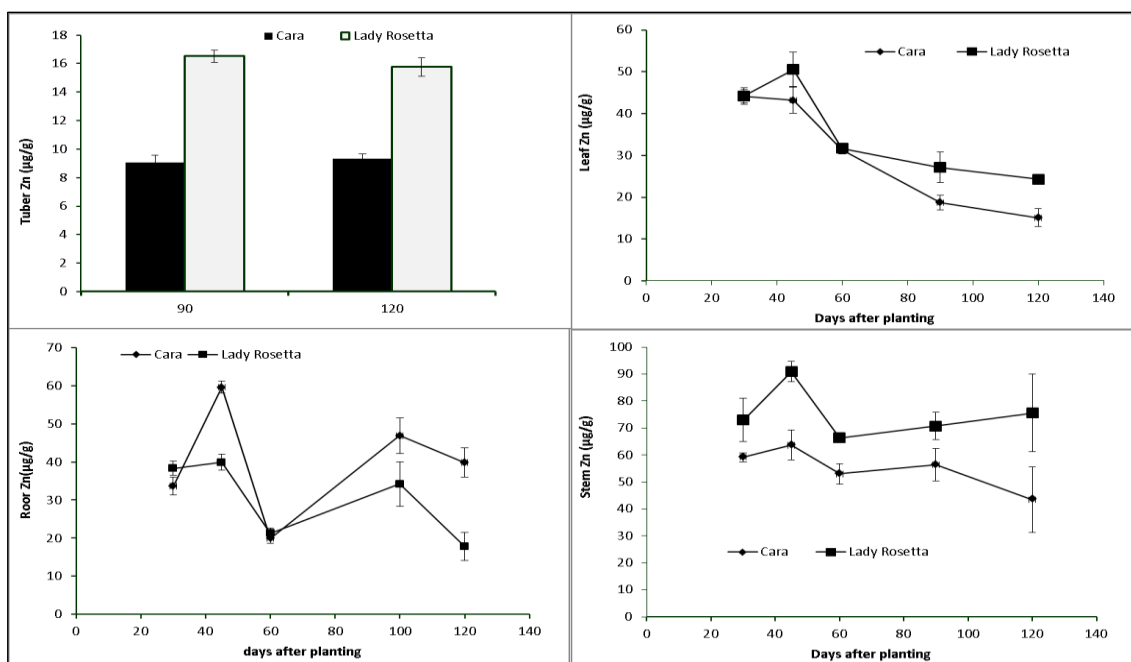


Figure 8.4 Zn concentrations in different organs of Cara and Lady Rosetta over time. Data points represent the mean+ standard error of the mean (n=3), 2015 data.

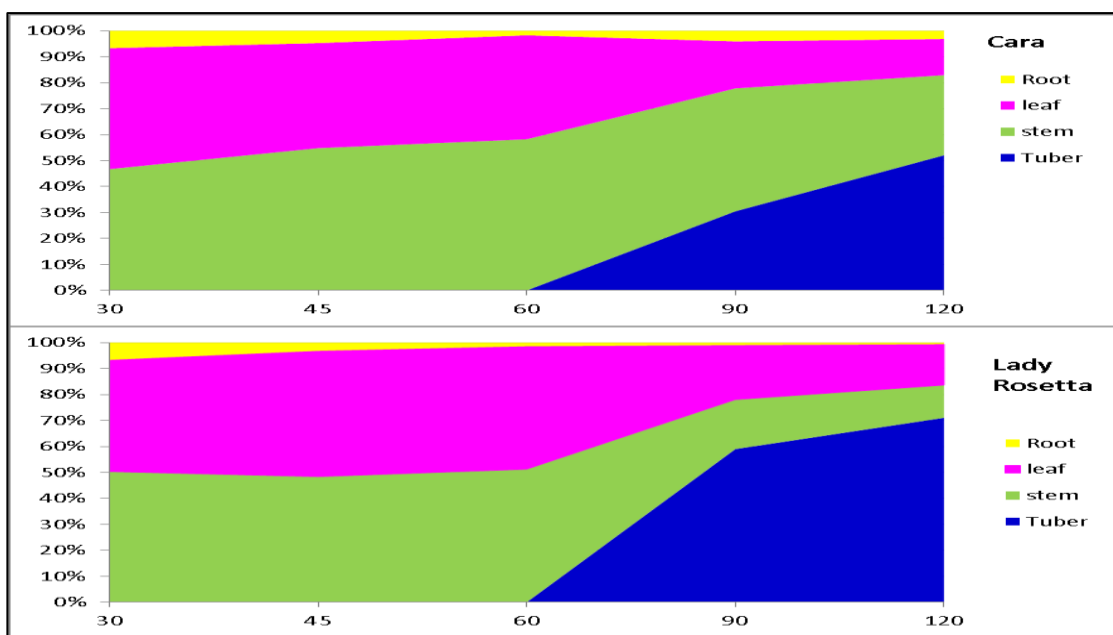


Figure 8.5 Distribution of Zn in all organs of potato plants over growth period.

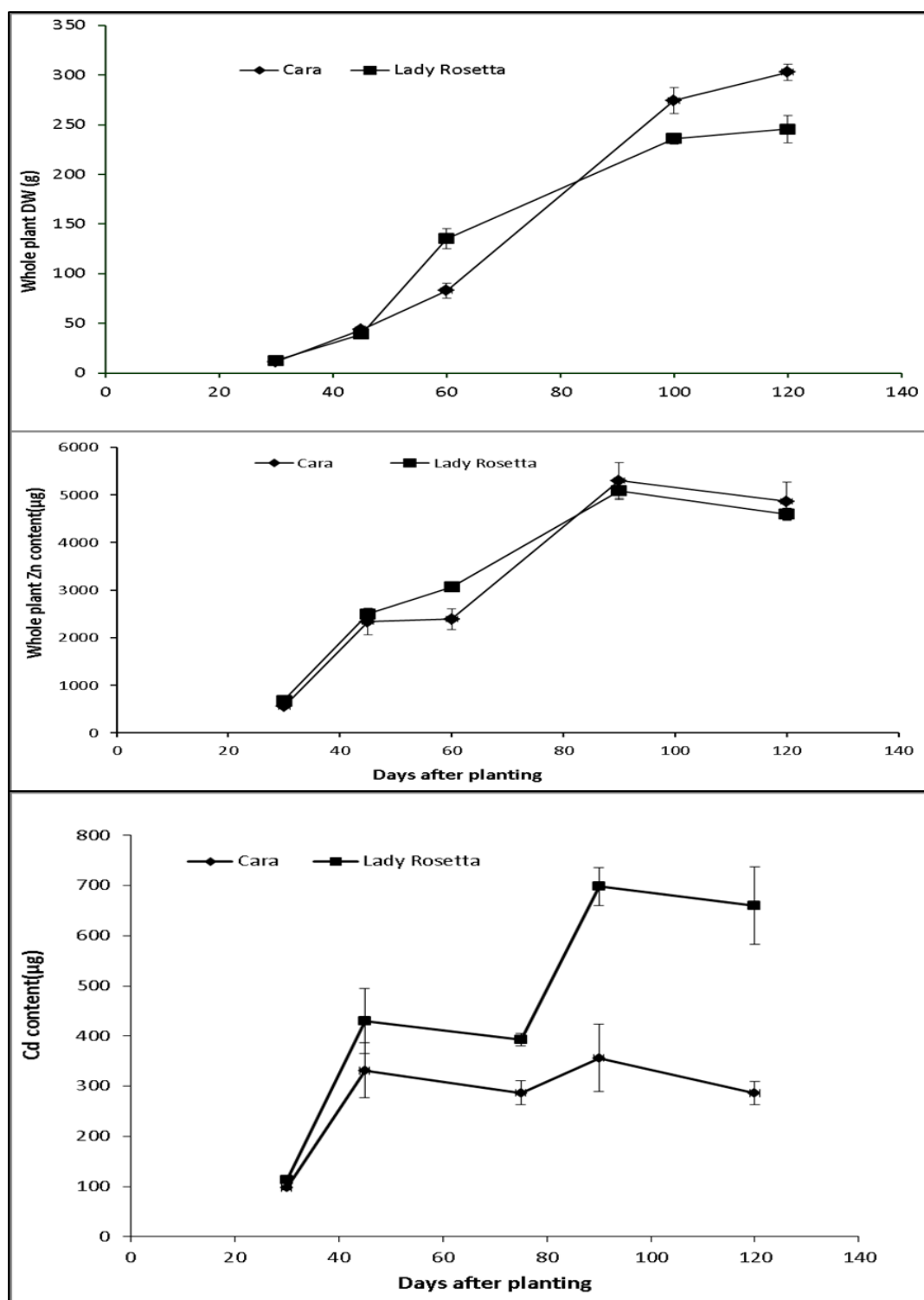


Figure 8.6 Total dry weight, Zn and Cd content per plant over the growth period. Values are mean±standard error of three plants (n=3).

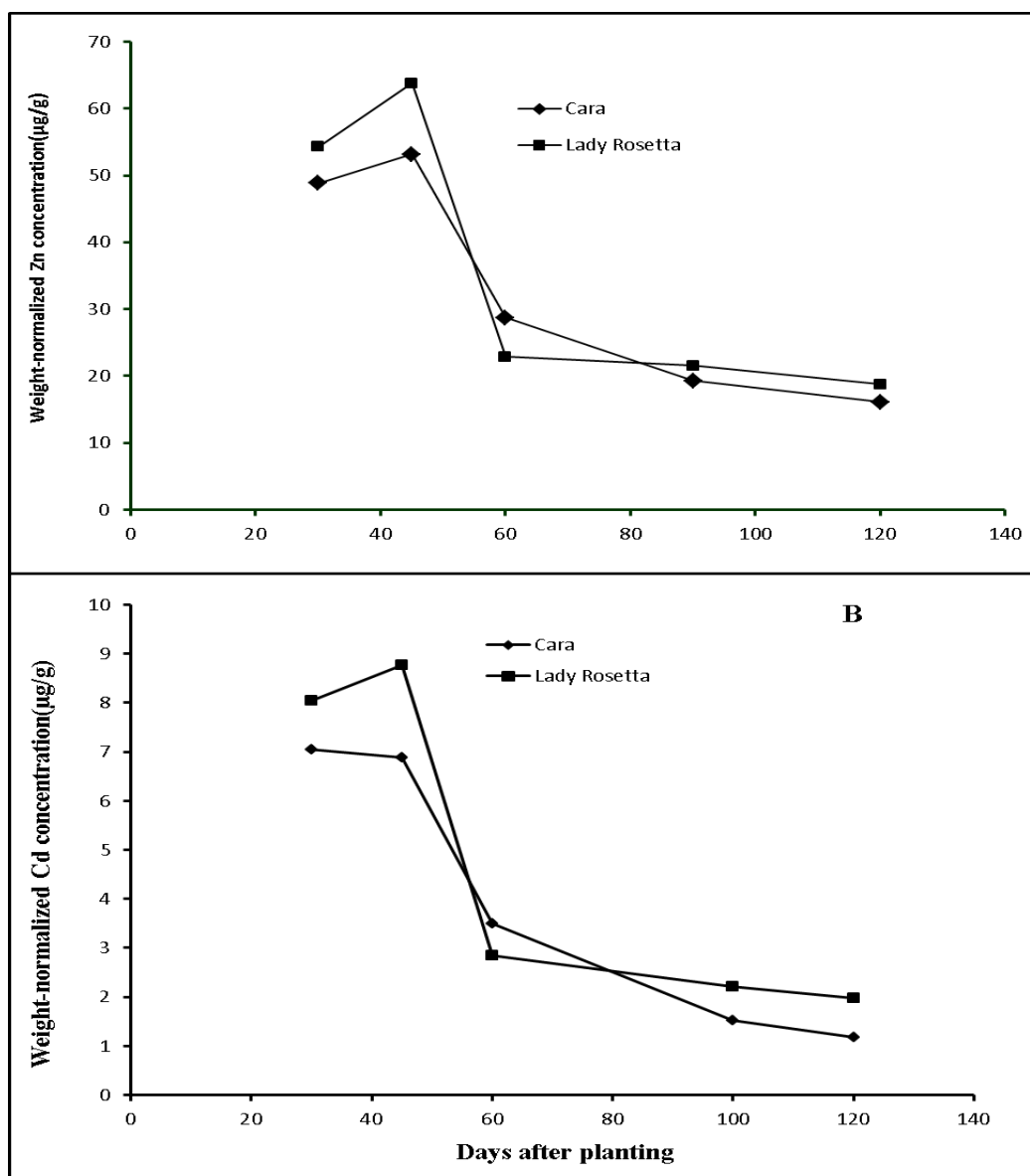


Figure 8.7 Weight-normalized whole plant Zn (A) and Cd (B) concentrations over the growth period. Values are the mean of three plants (n=3).

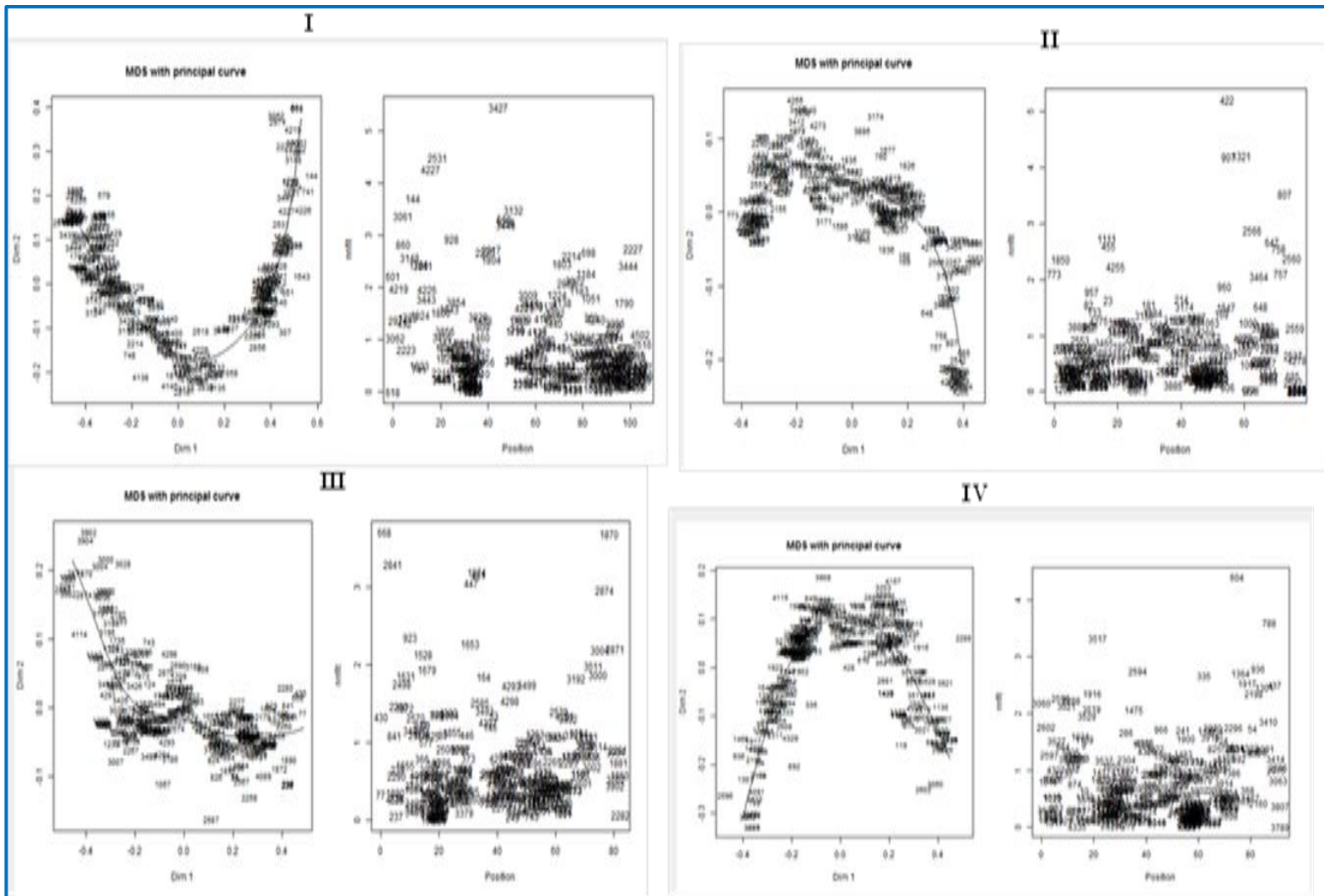


Figure 8.8 Multidimensional scaling (MDS) configuration for SNP markers of chromosome I to IV from cluster analysis.

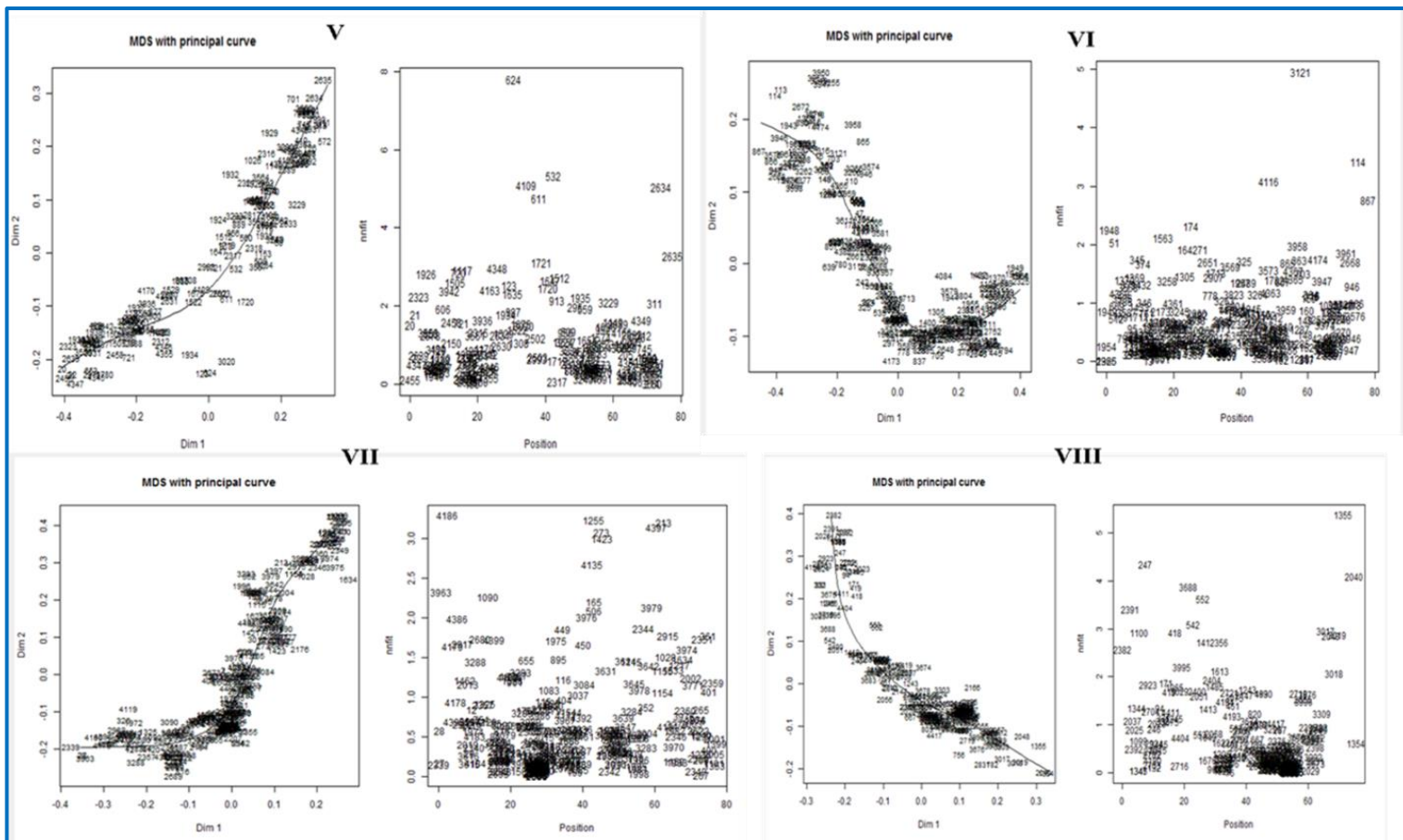


Figure 8.8 Multidimensional scaling configurations for SNP markers of chromosome V to VIII from the cluster analysis.

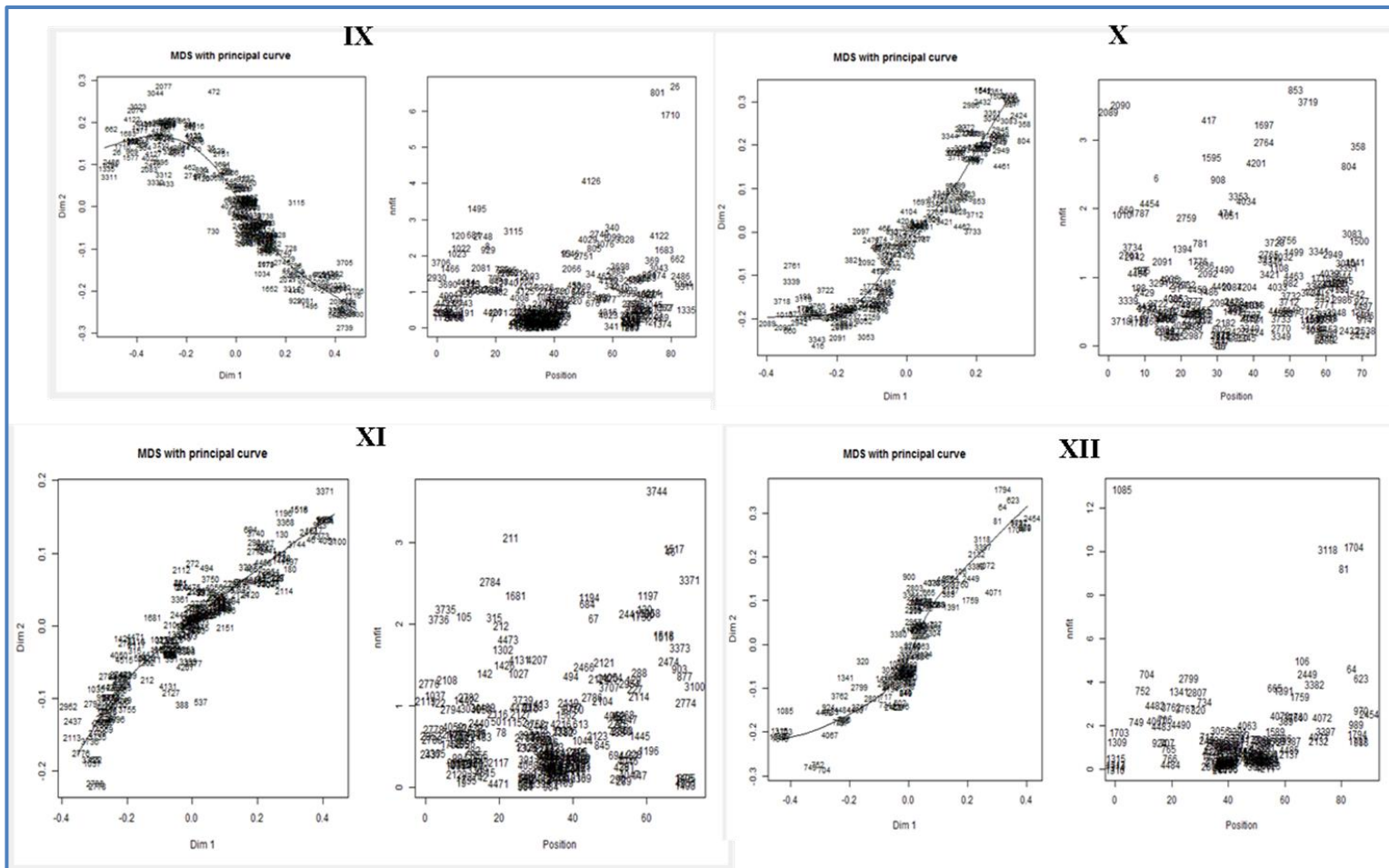


Figure 8.8 Multidimensional scaling configurations for SNP markers of chromosome IX to XII from the cluster analysis.

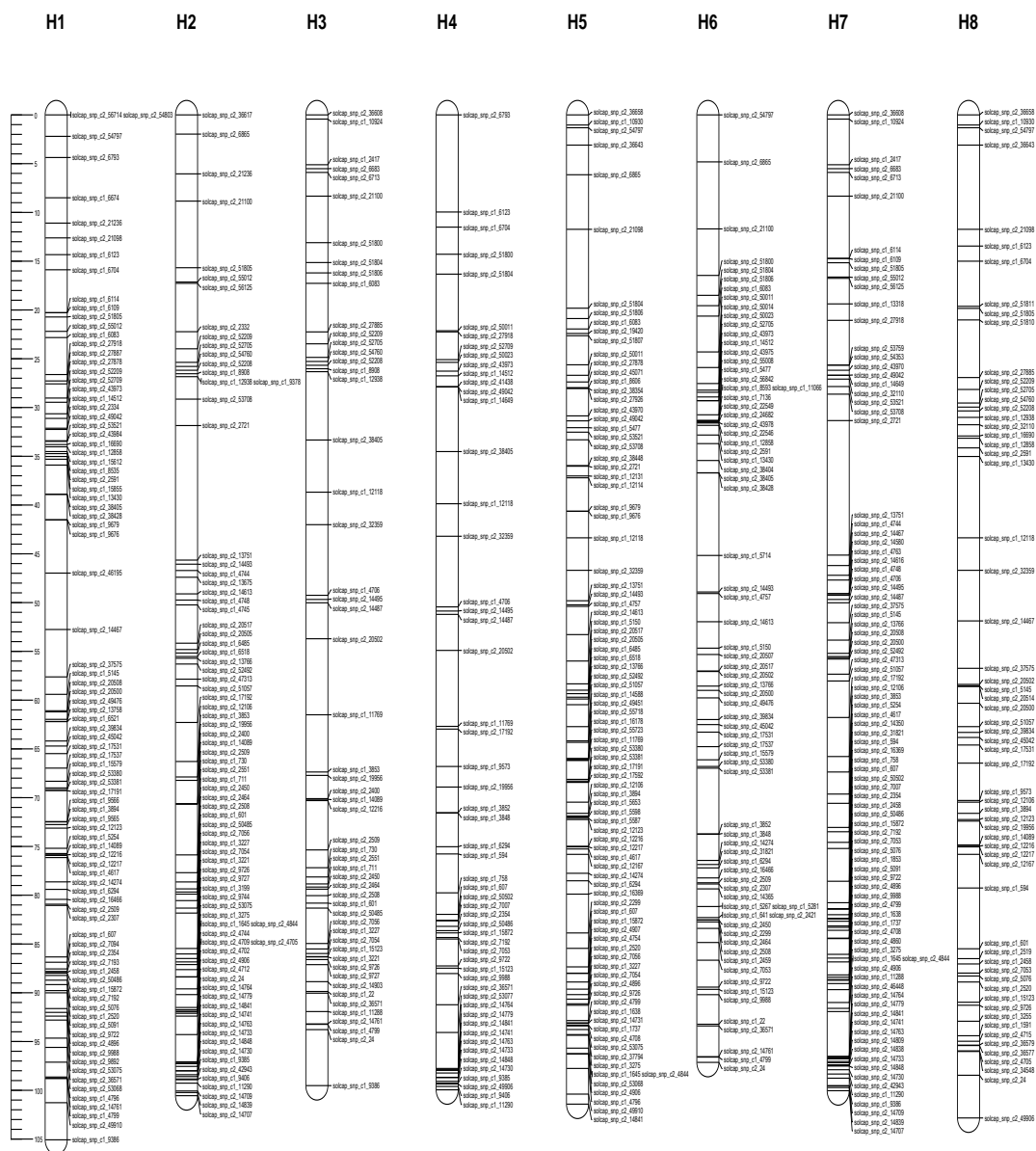


Figure 8.9 Distribution of markers across eight homologues of chromosome I with the first four homologues from Lady Rosetta (H1 to H4) and Cara (H5 to H8).

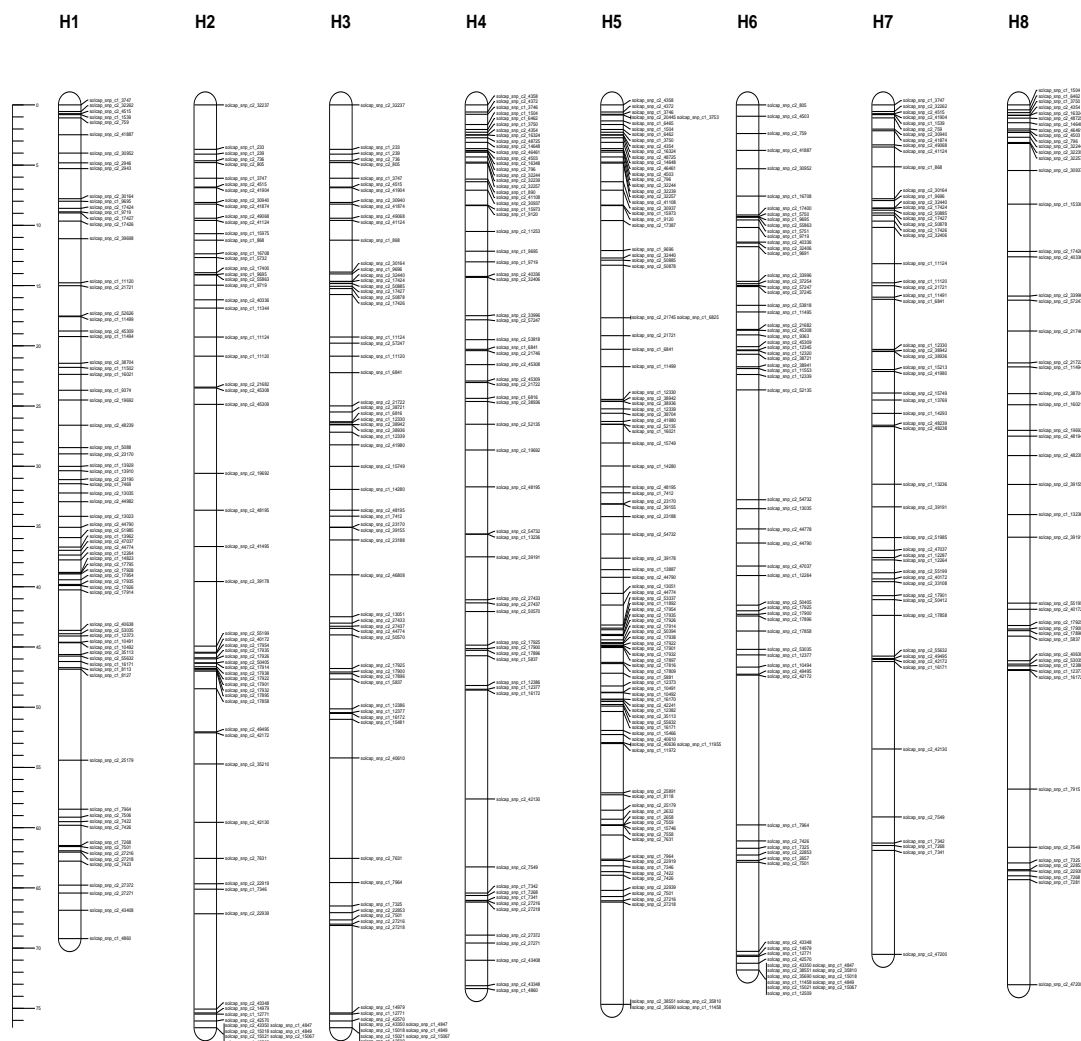


Figure 8.9 Distribution of markers across eight homologues of chromosome **II** with the first four homologues from Lady Rosetta (H1 to H4) and Cara (H5 to H8).

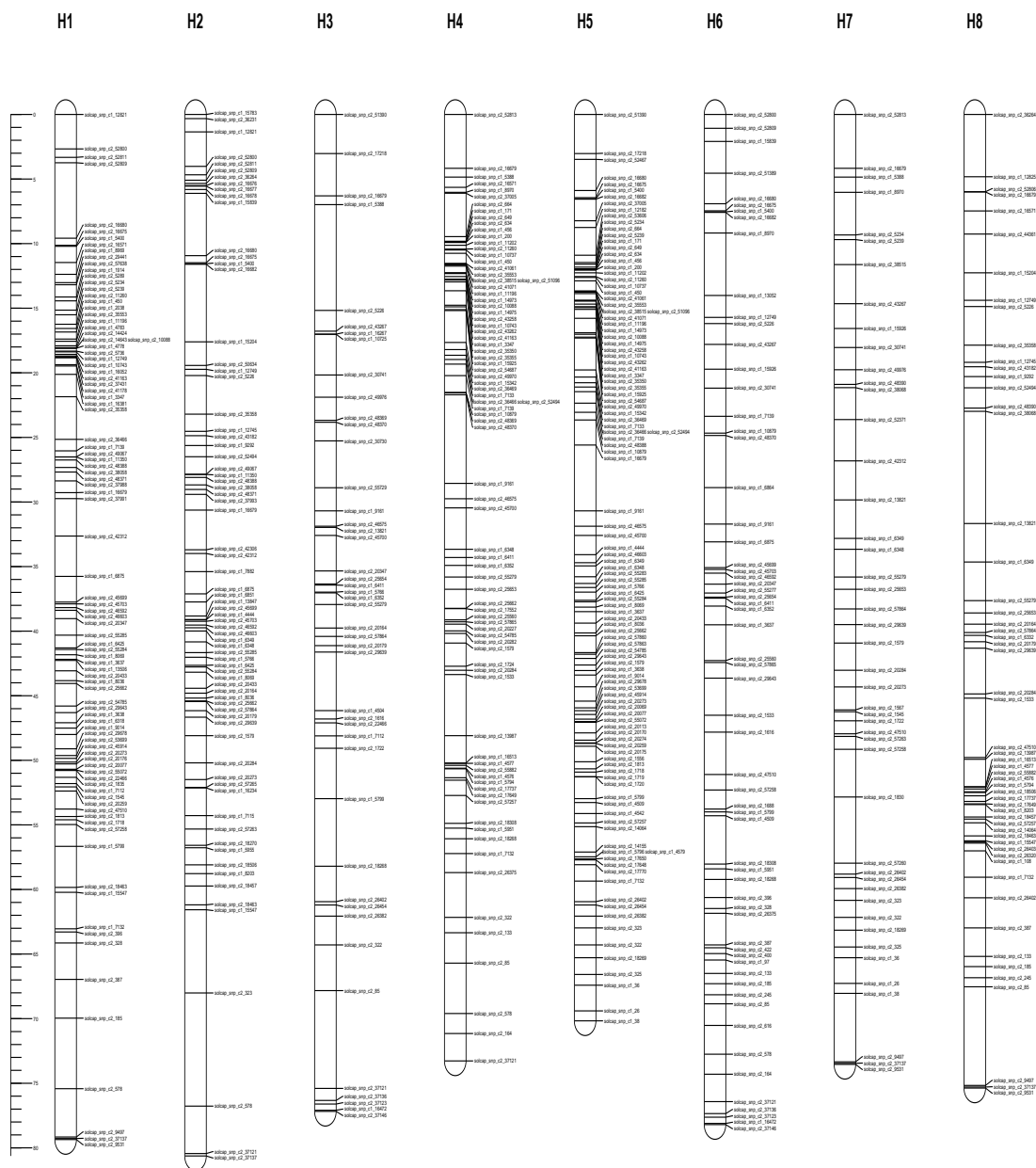


Figure 8.9 Distribution of markers across eight homologues of chromosome **III** with the first four homologues from Lady Rosetta (H1 to H4) and Cara (H5 to H8).

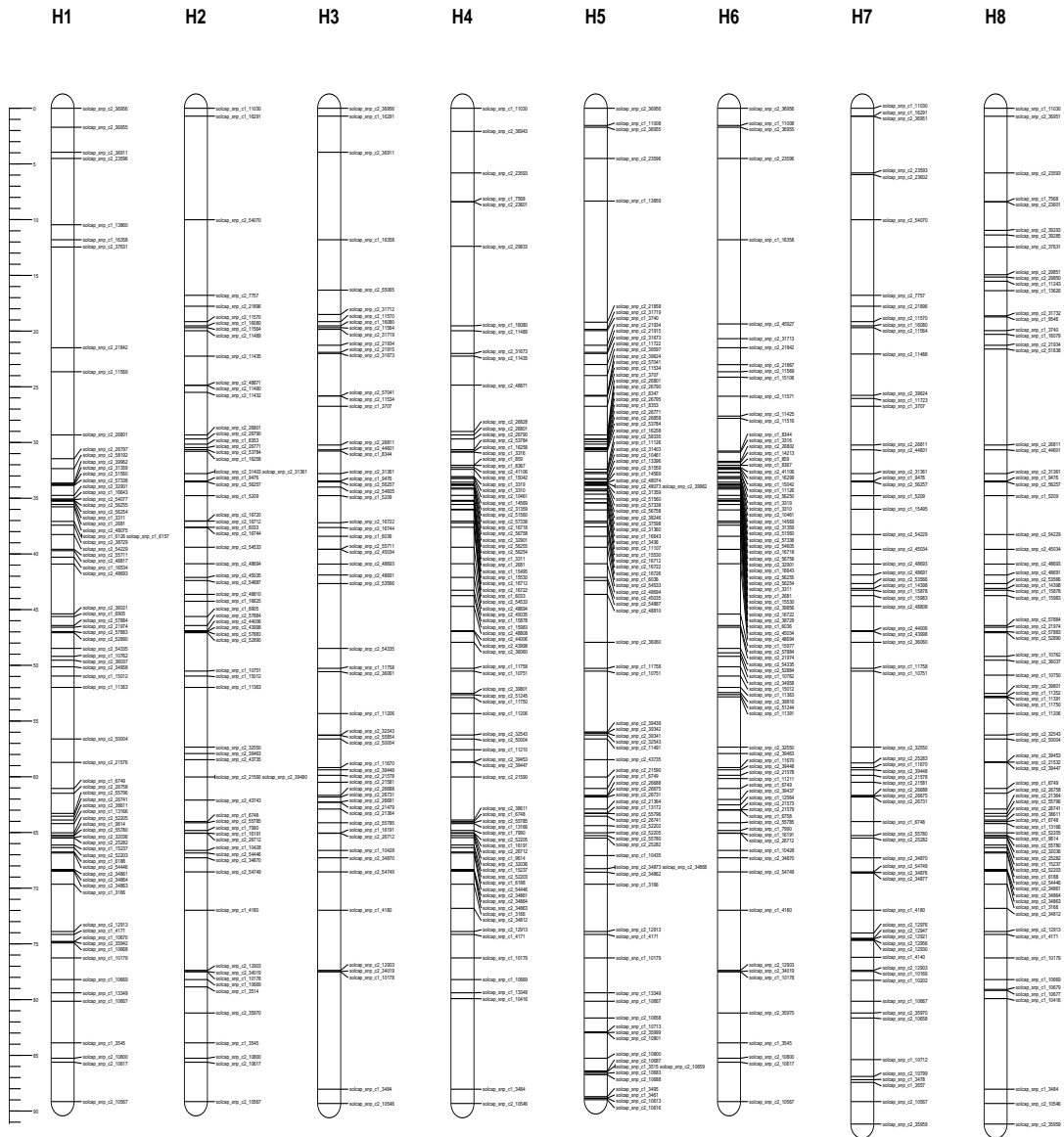


Figure 8.9 Distribution of markers across eight homologues of chromosome **IV** with the first four homologues from Lady Rosetta (H1 to H4) and Cara (H5 to H8).

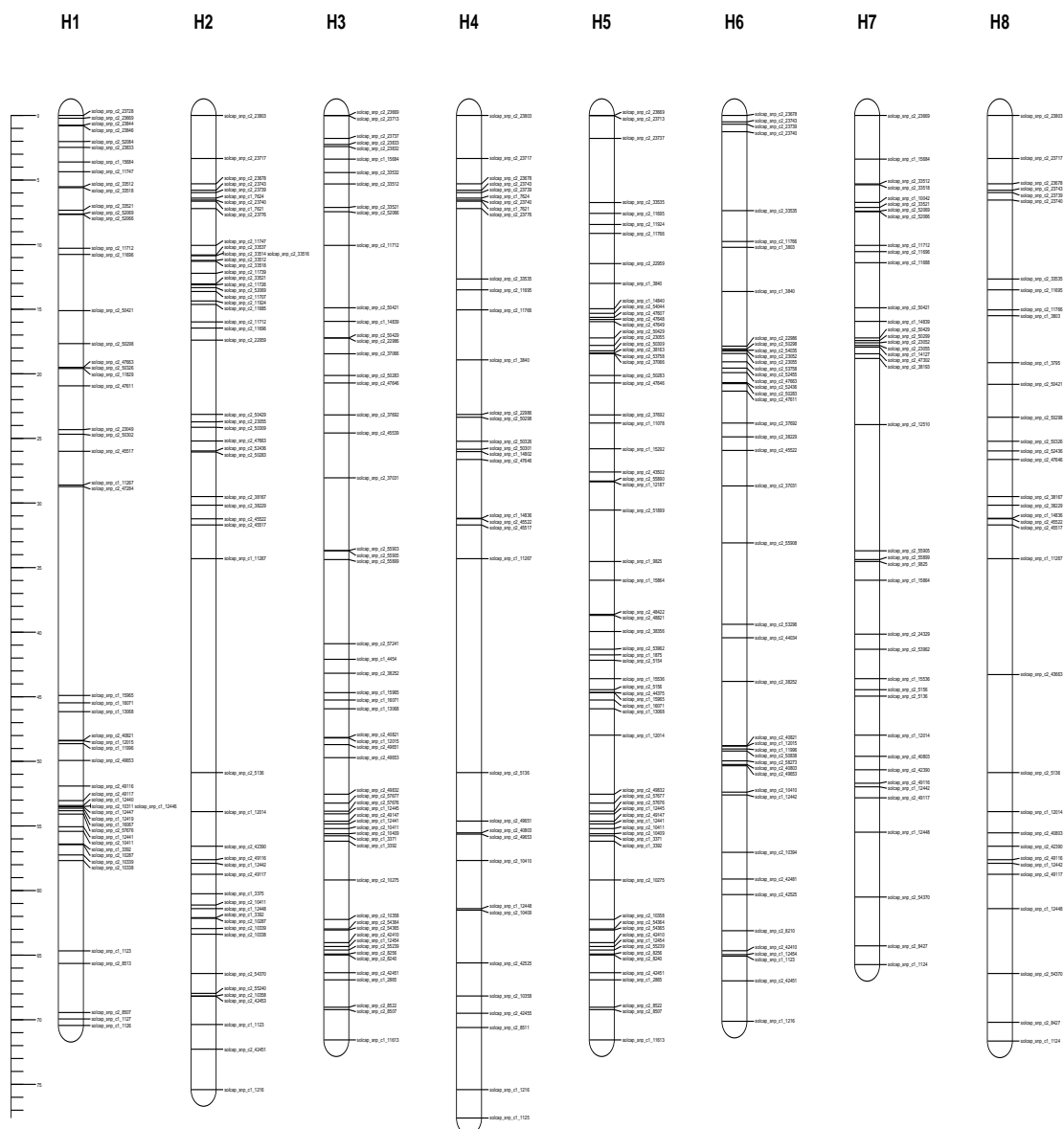


Figure 8.9 Distribution of markers across eight homologues of chromosome V with the first four homologues from Lady Rosetta (H1 to H4) and Cara (H5 to H8).

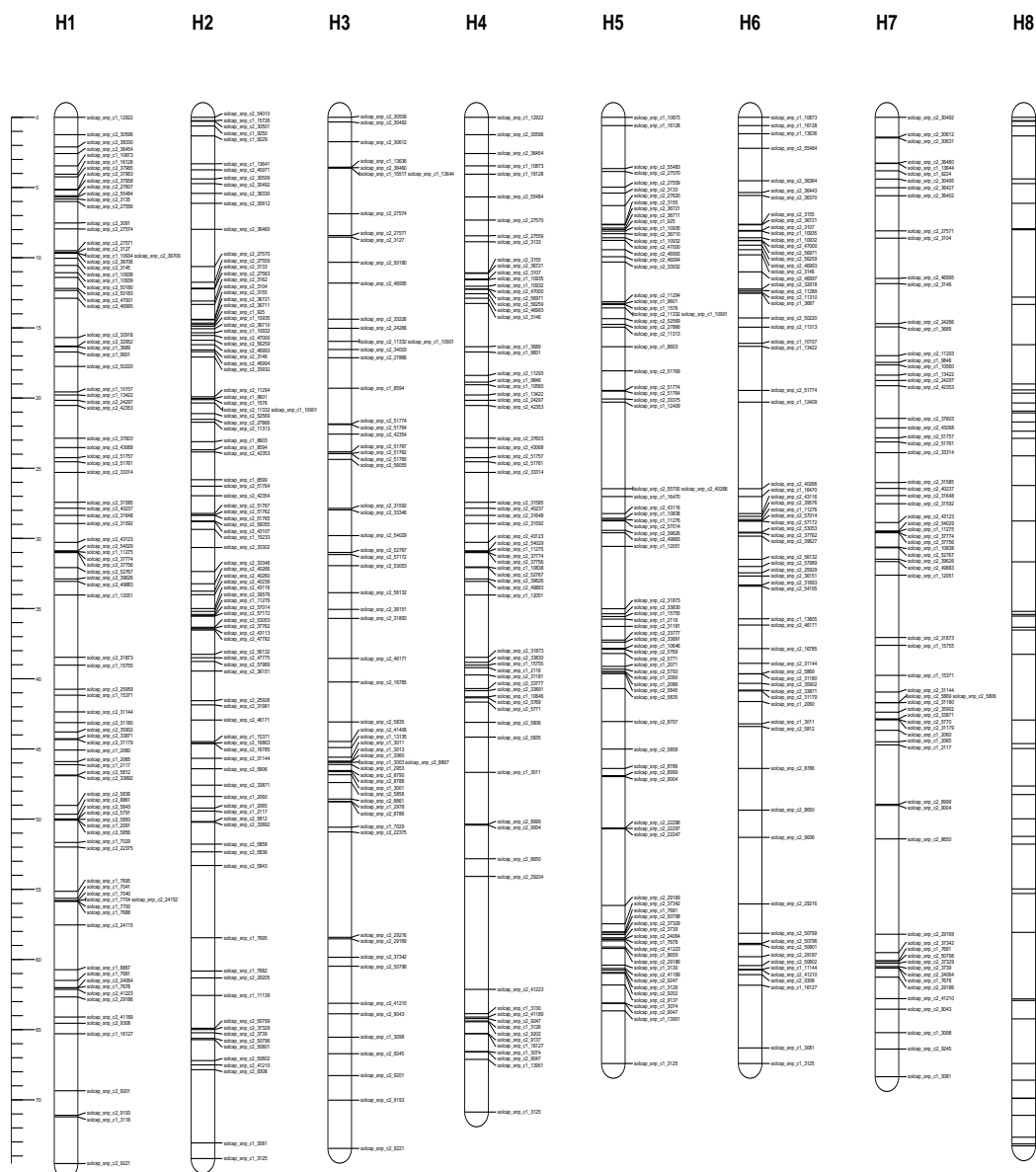


Figure 8.9 Distribution of markers across eight homologues of chromosome **VI** with the first four homologues from Lady Rosetta (H1 to H4) and Cara (H5 to H8).

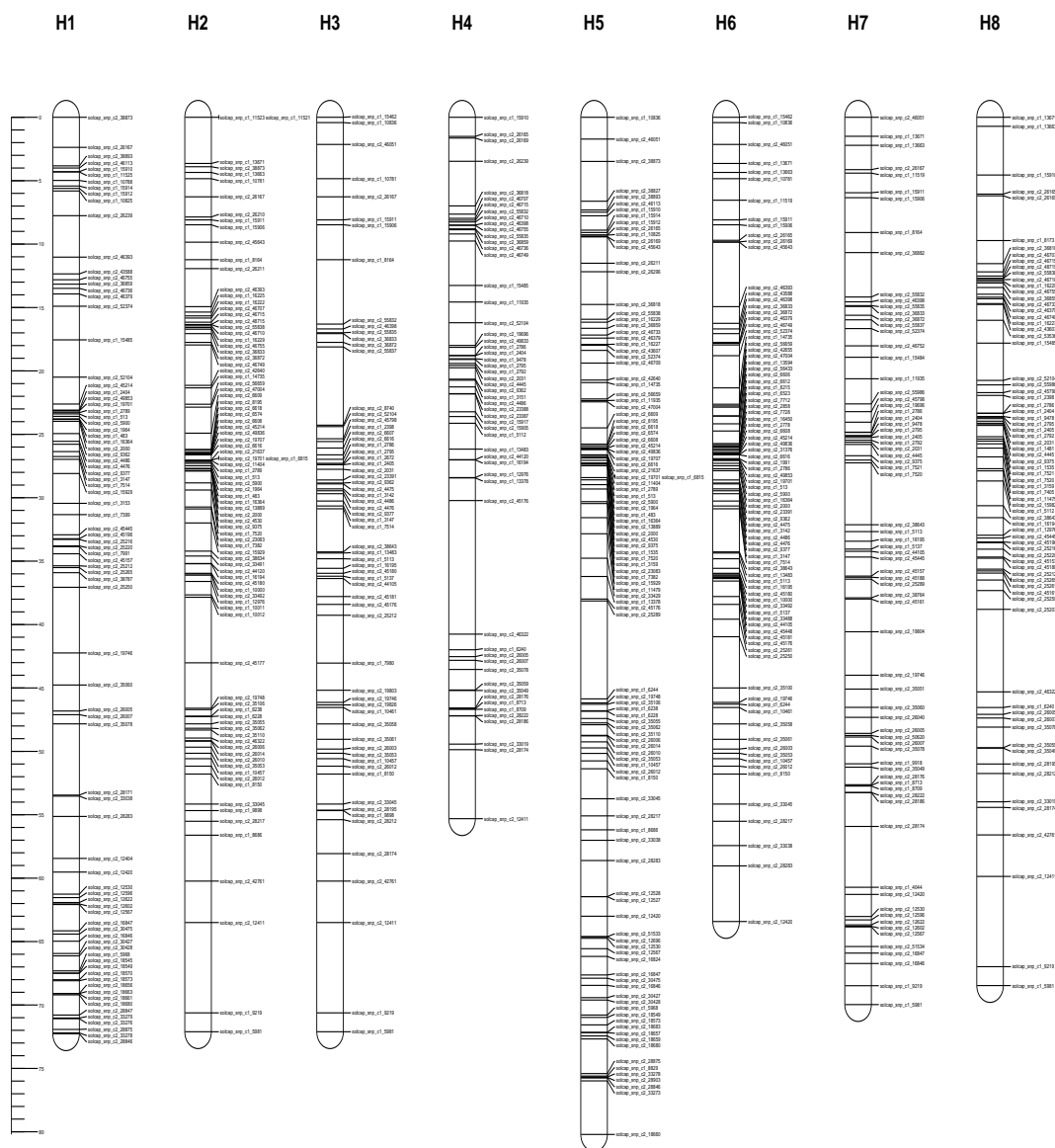


Figure 8.9 Distribution of markers across eight homologues of chromosome **VII** with the first four homologues from Lady Rosetta (H1 to H4) and Cara (H5 to H8).

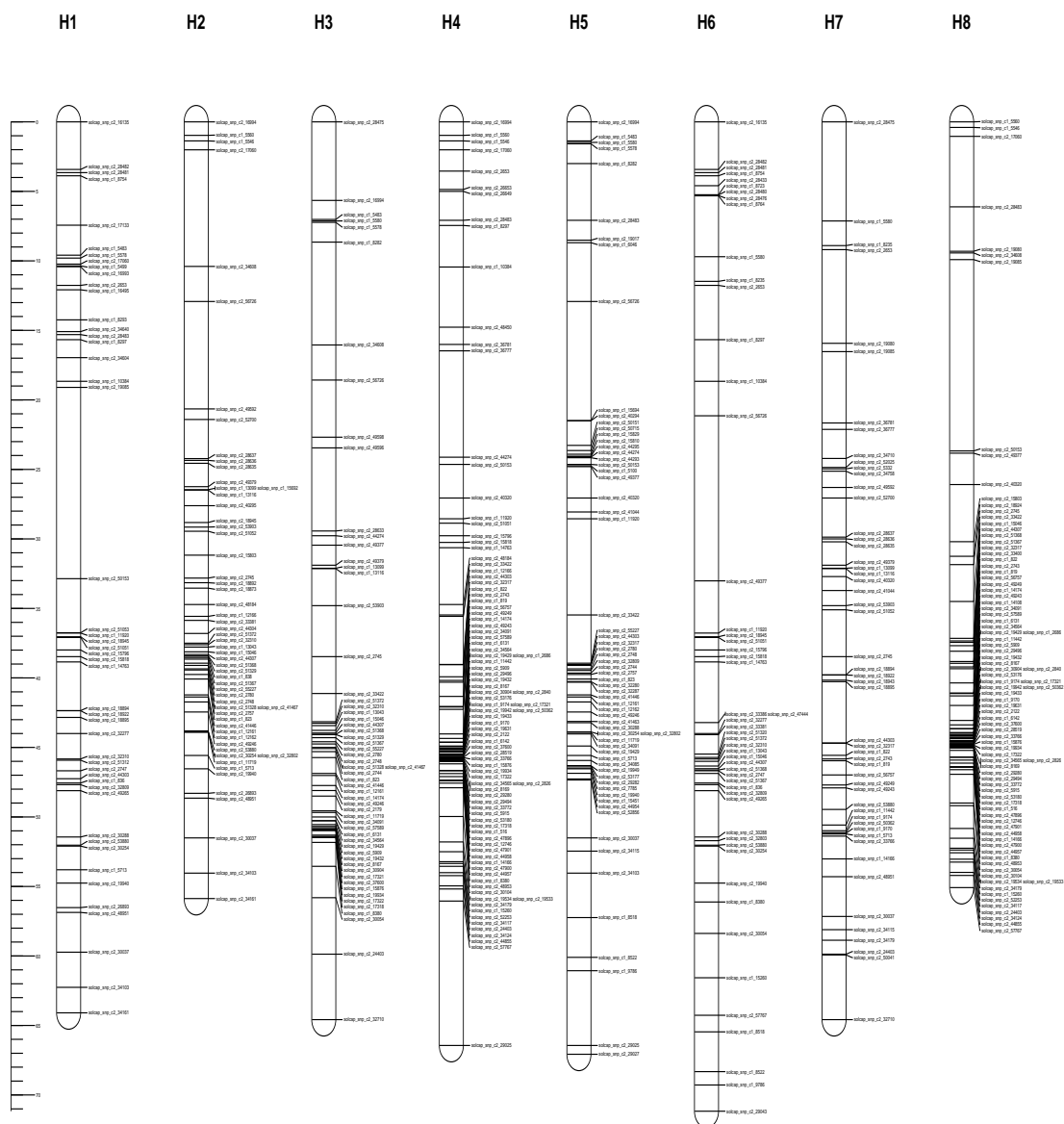


Figure 8.9 Distribution of markers across eight homologues of chromosome **VIII** with the first four homologues from Lady Rosetta (H1 to H4) and Cara (H5 to H8).

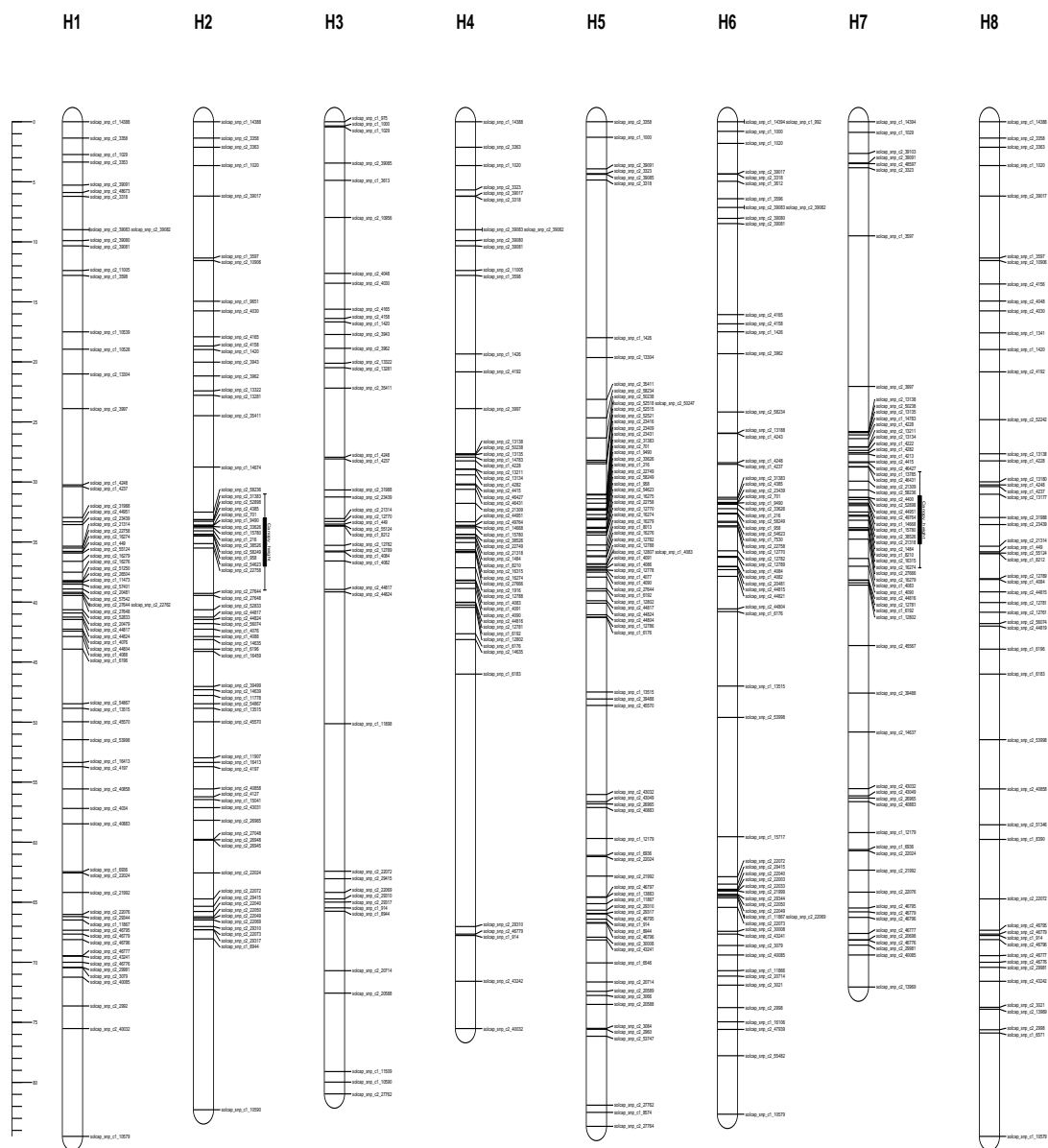


Figure 8.9 Distribution of markers across eight homologues of chromosome **IX** with the first four homologues from Lady Rosetta (H1 to H4) and Cara (H5 to H8).

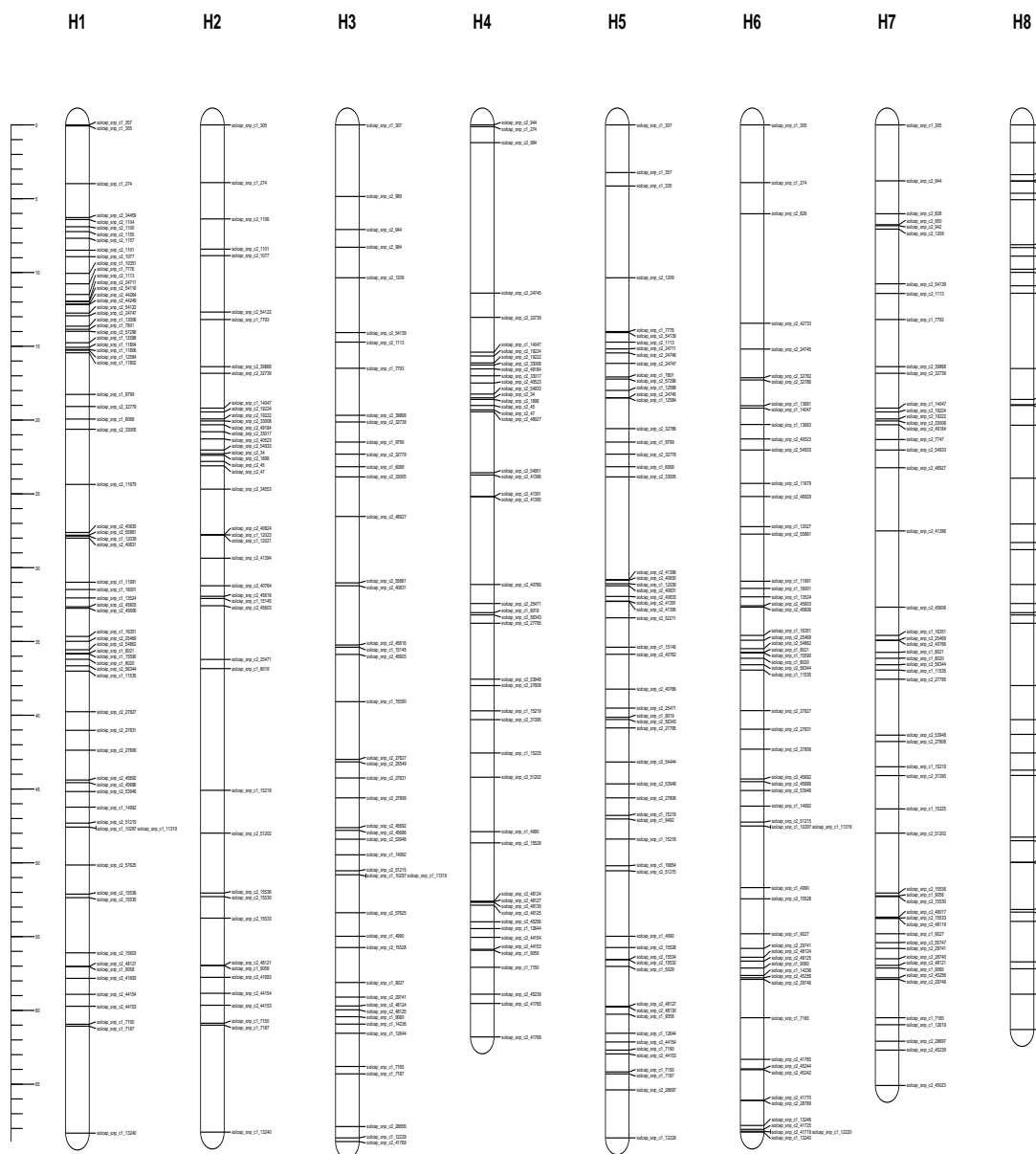


Figure 8.9 Distribution of markers across eight homologues of chromosome X with the first four homologues from Lady Rosetta (H1 to H4) and Cara (H5 to H8).

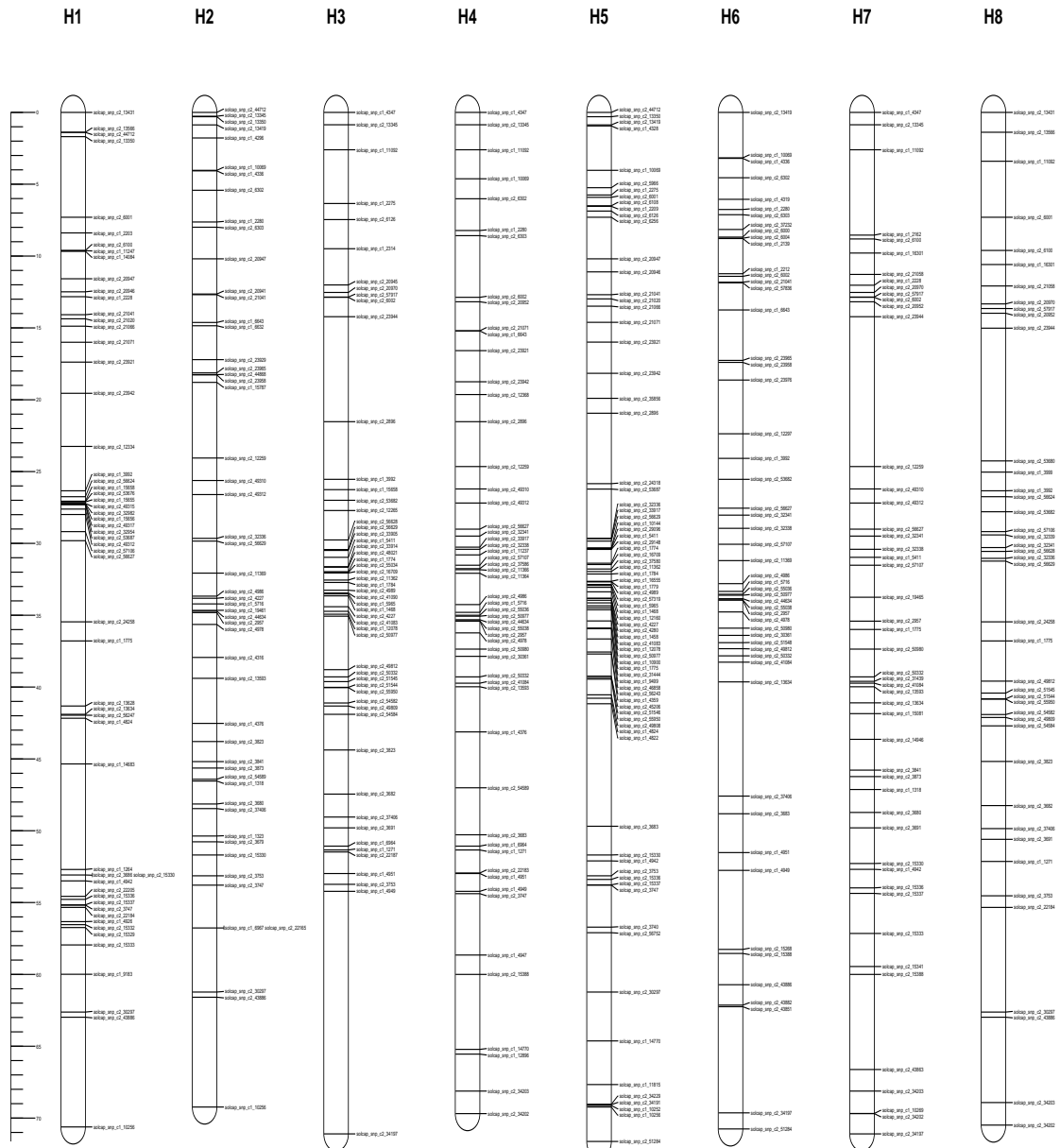


Figure 8.9 Distribution of markers across eight homologues of chromosome **XI** with the first four homologues from Lady Rosetta (H1 to H4) and Cara (H5 to H8).

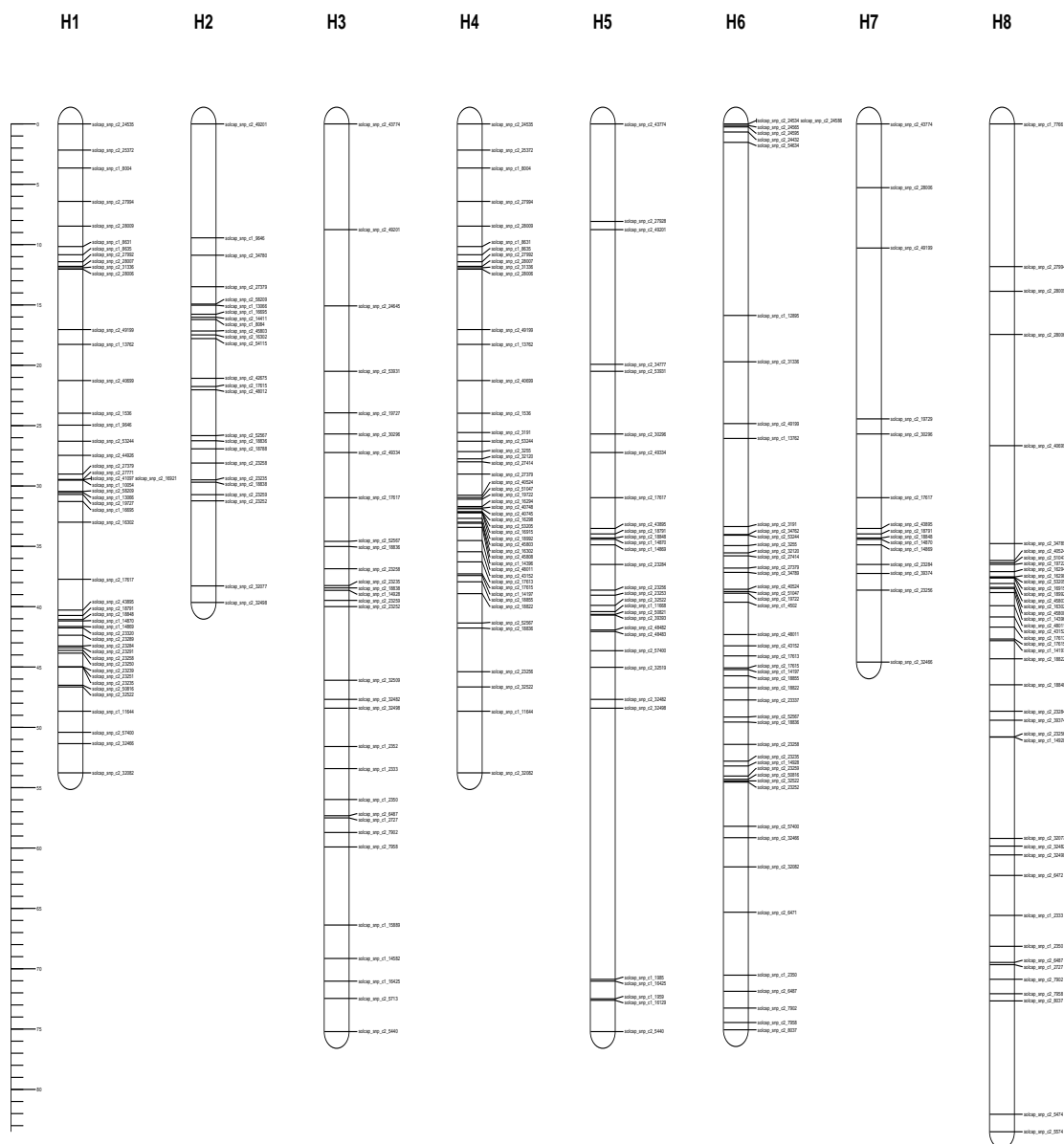


Figure 8.9 Distribution of markers across eight homologues of chromosome **XII** with the first four homologues from Lady Rosetta (H1 to H4) and Cara (H5 to H8).

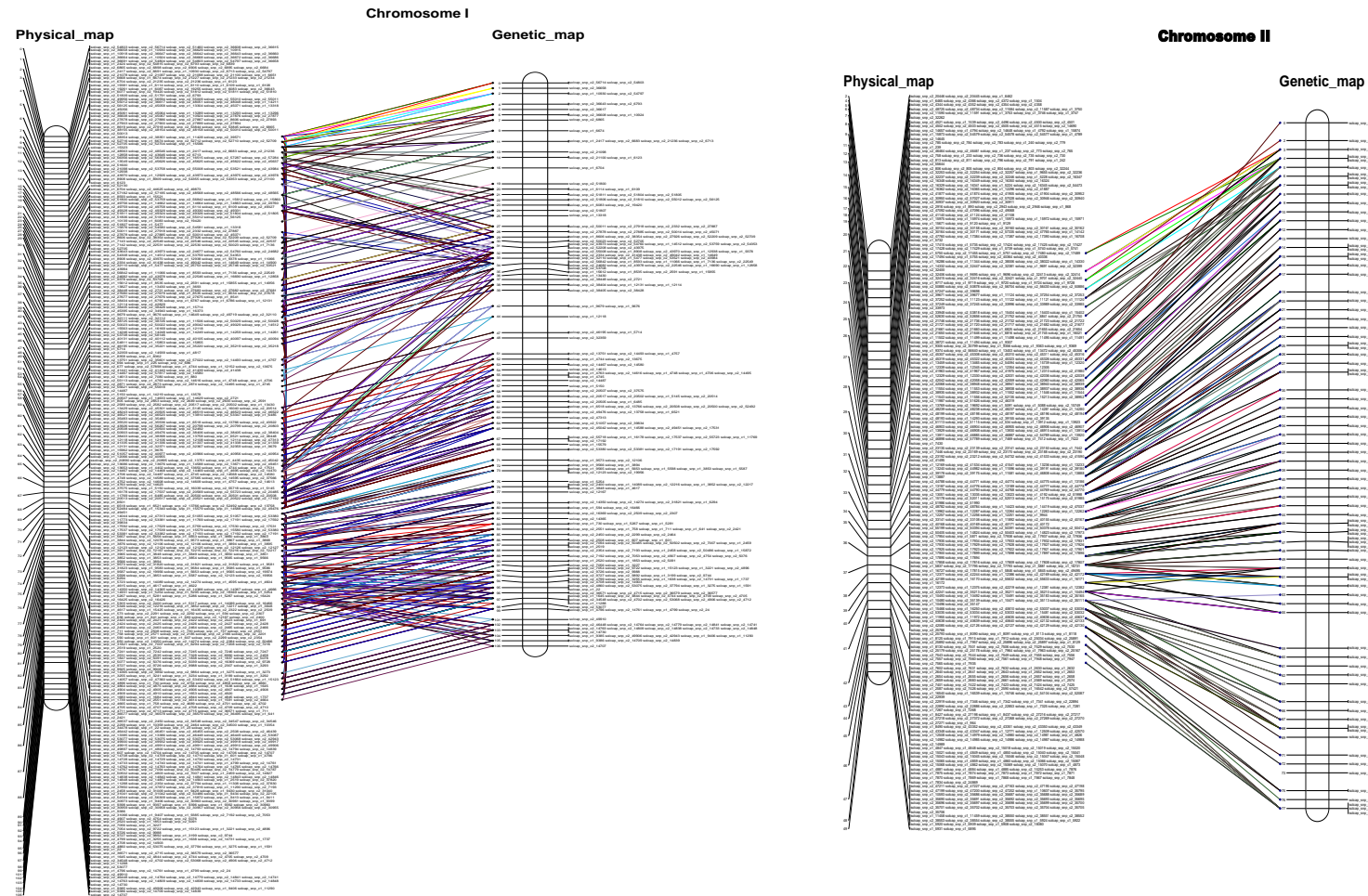


Figure 8.10 Visualization of the agreement between the physical map(Mb) and the genetic map (cM) alignments of Chromosome I (left) and chromosome II (right).

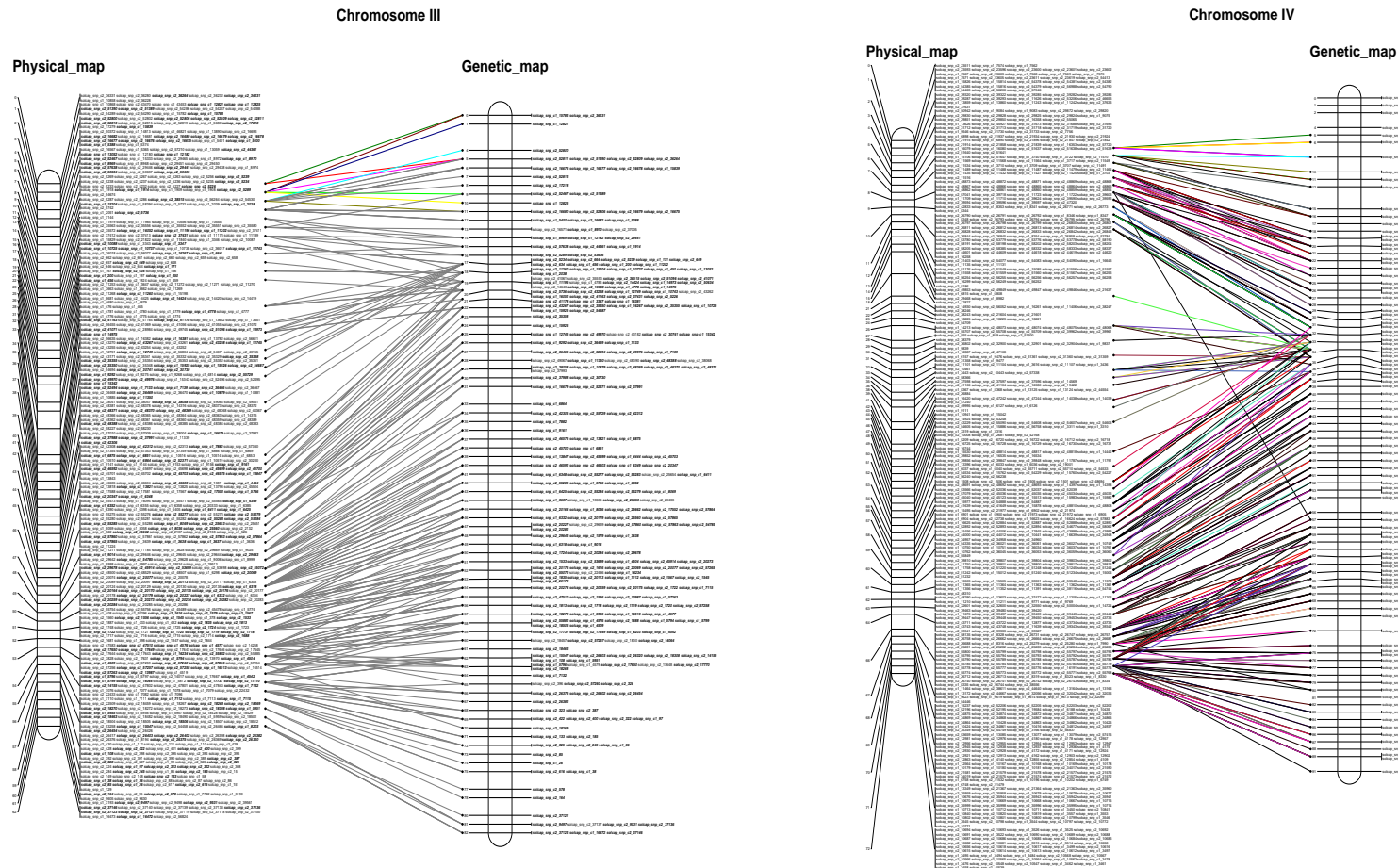


Figure 8.10 Visualization of the agreement between the physical map (Mb) and the genetic map (cM) alignments of Chromosome III (left) and chromosome IV (right).

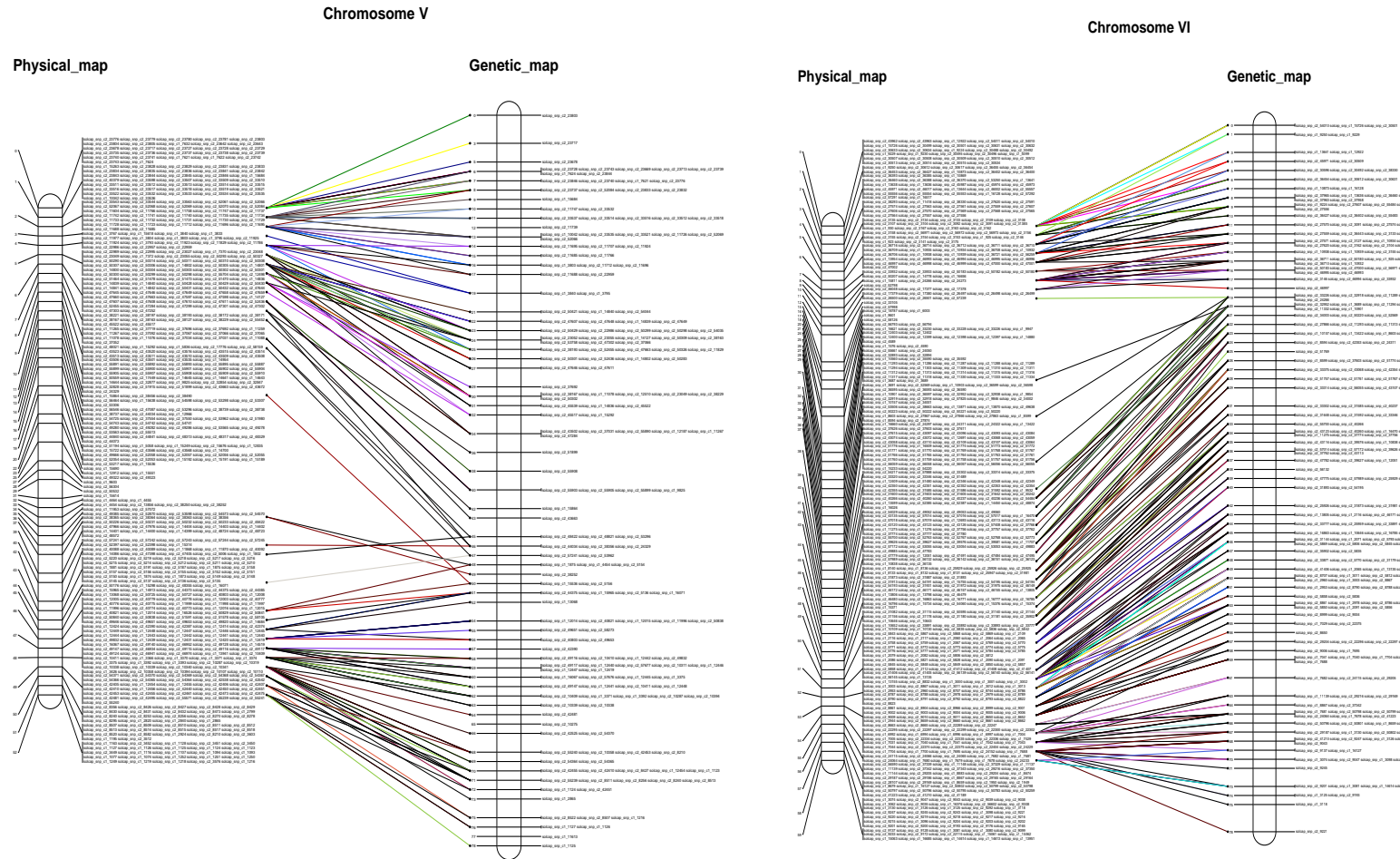


Figure 8.10 Visualization of the agreement between the physical map(Mb) and the genetic map (cM) alignments of Chromosome V (left) and chromosome VI (right).

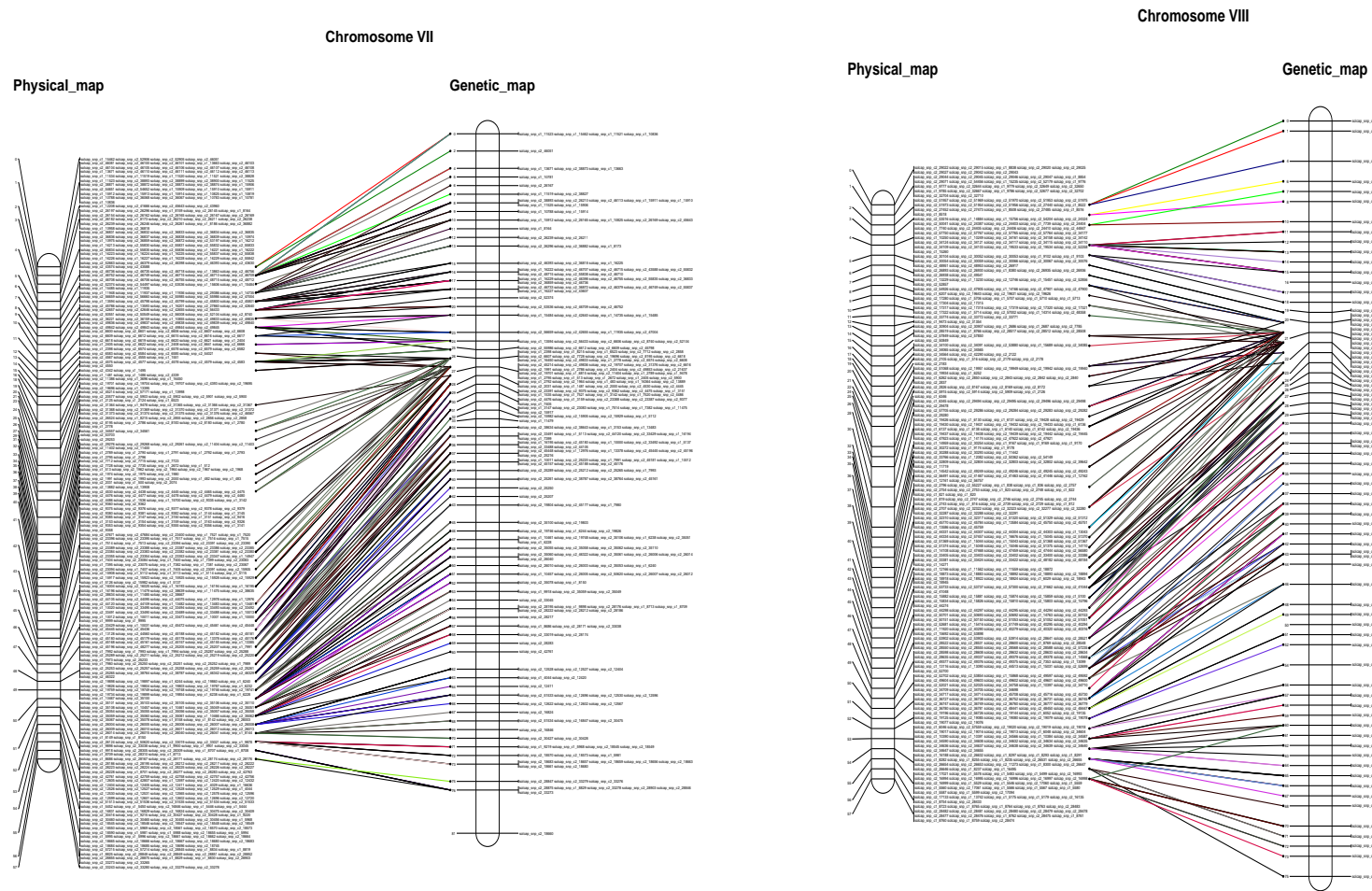


Figure 8.10 Visualization of the agreement between the physical map (Mb) and the genetic map (cM) alignments of Chromosome VII (left) and chromosome VIII (right).

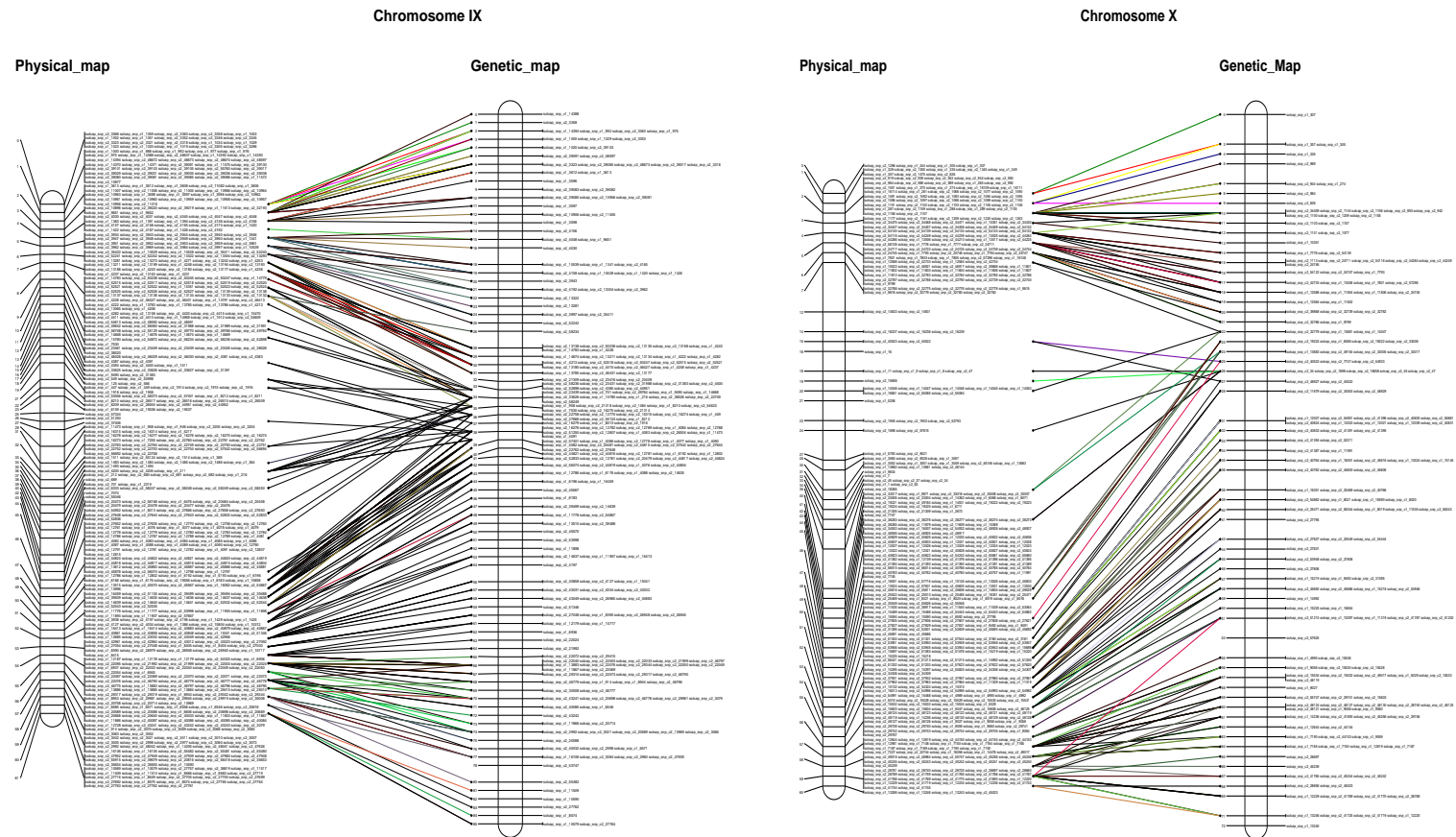


Figure 8.10 Visualization of the agreement between the physical map (Mb) and the genetic map (cM) alignments of Chromosome IX (left) and chromosome X (right).

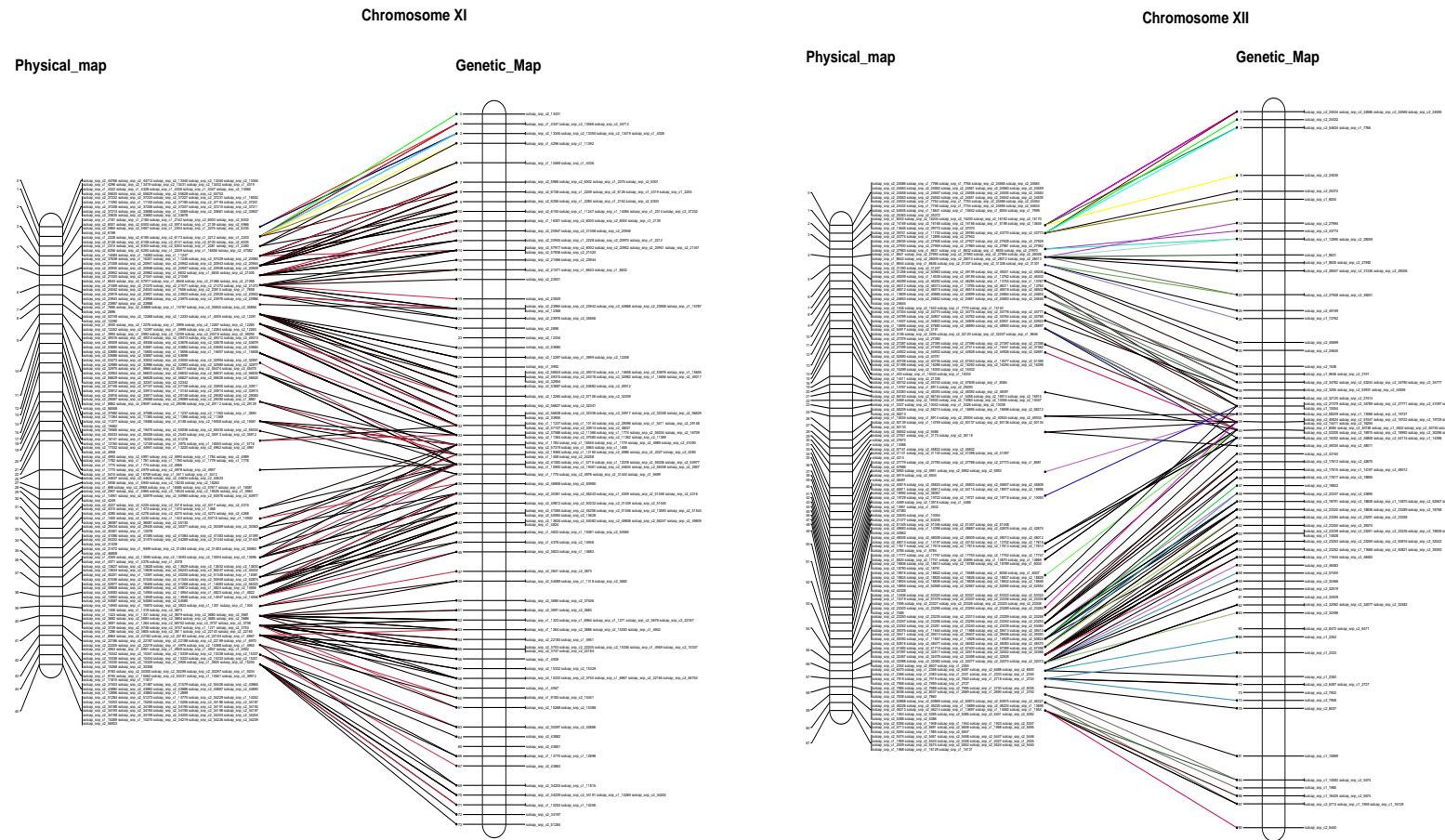


Figure 8.10 Visualization of the agreement between the physical map (Mb) and the genetic map (cM) alignments of Chromosome XI (left) and chromosome XII (right).

Chromosome I

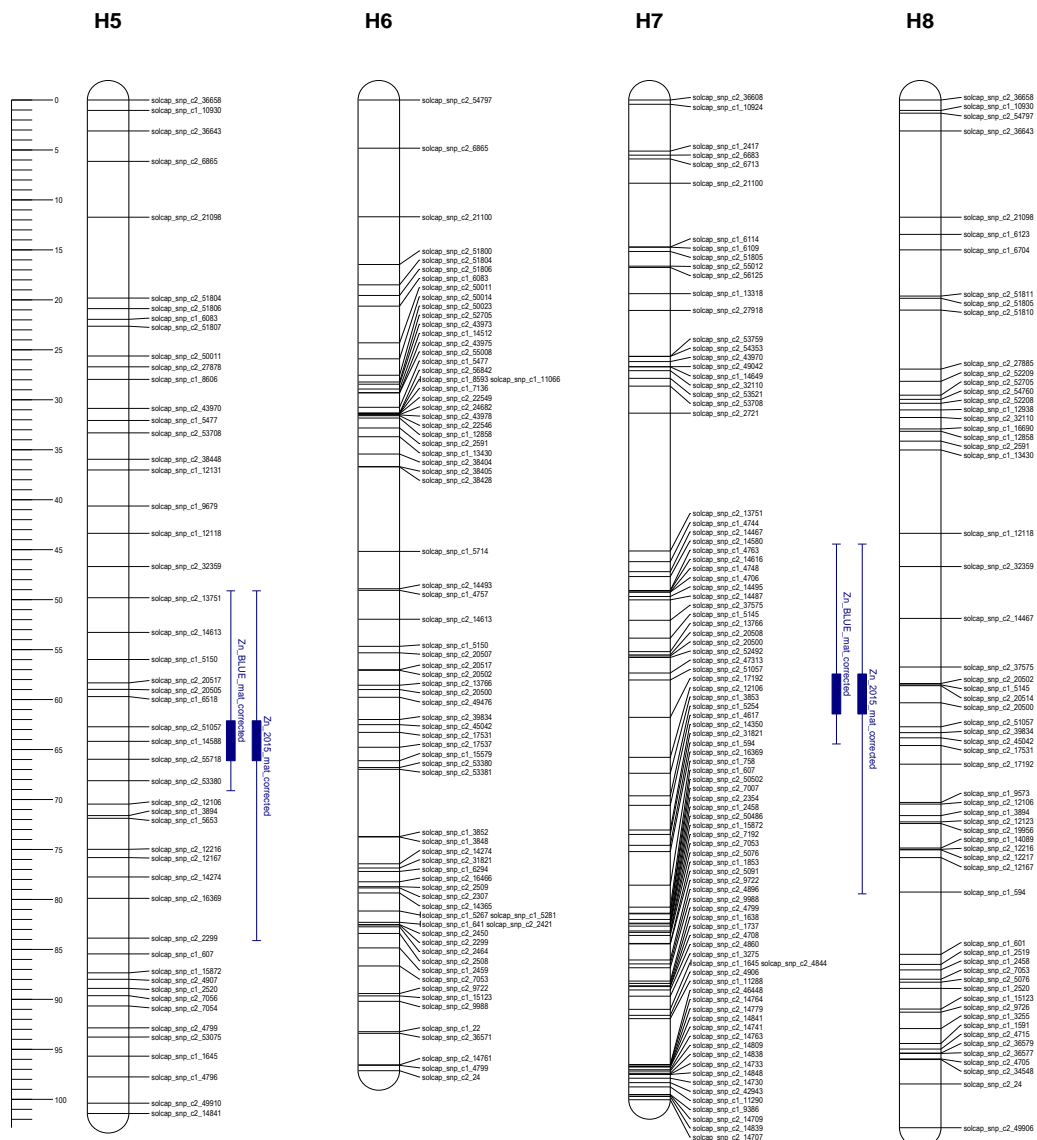


Figure 8.11 Linkage map of Cara chromosome I (H5 to H8=homologous maps). The blue boxplots represent the QTL position for tuber Zn concentration. In the boxplot, whiskers correspond to the two LOD support interval and the solid box represents the 95% confidence interval.

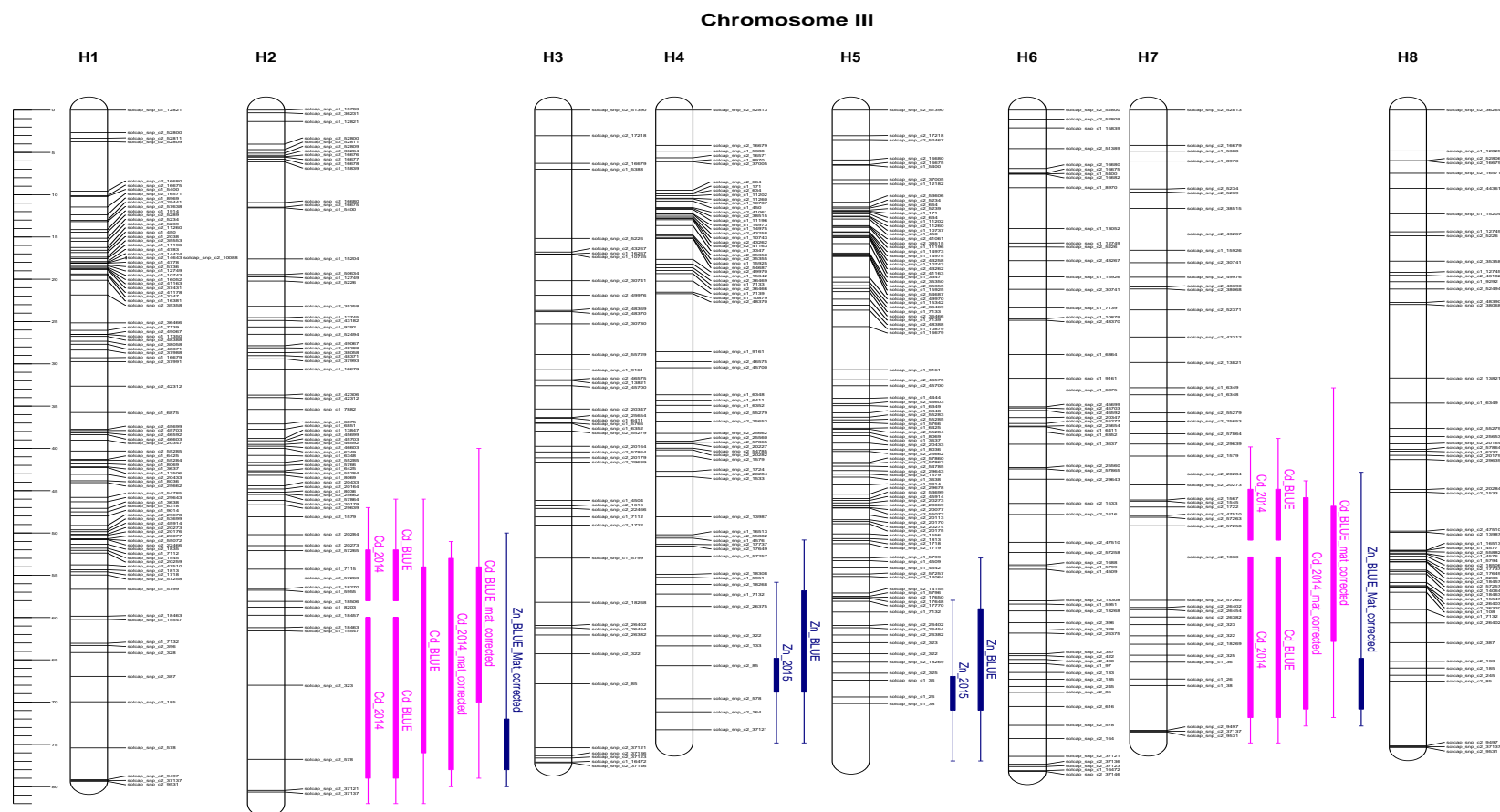


Figure 8.11 Linkage maps of Lady Rosetta (H1 to H4) and Cara (H5 to H8) chromosome III . The blue and rose boxplots correspond to the QTL position for tuber Cd and Zn concentration, respectively. In the boxplot, whiskers correspond to the two LOD support interval and the solid box represents 95% confidence interval.

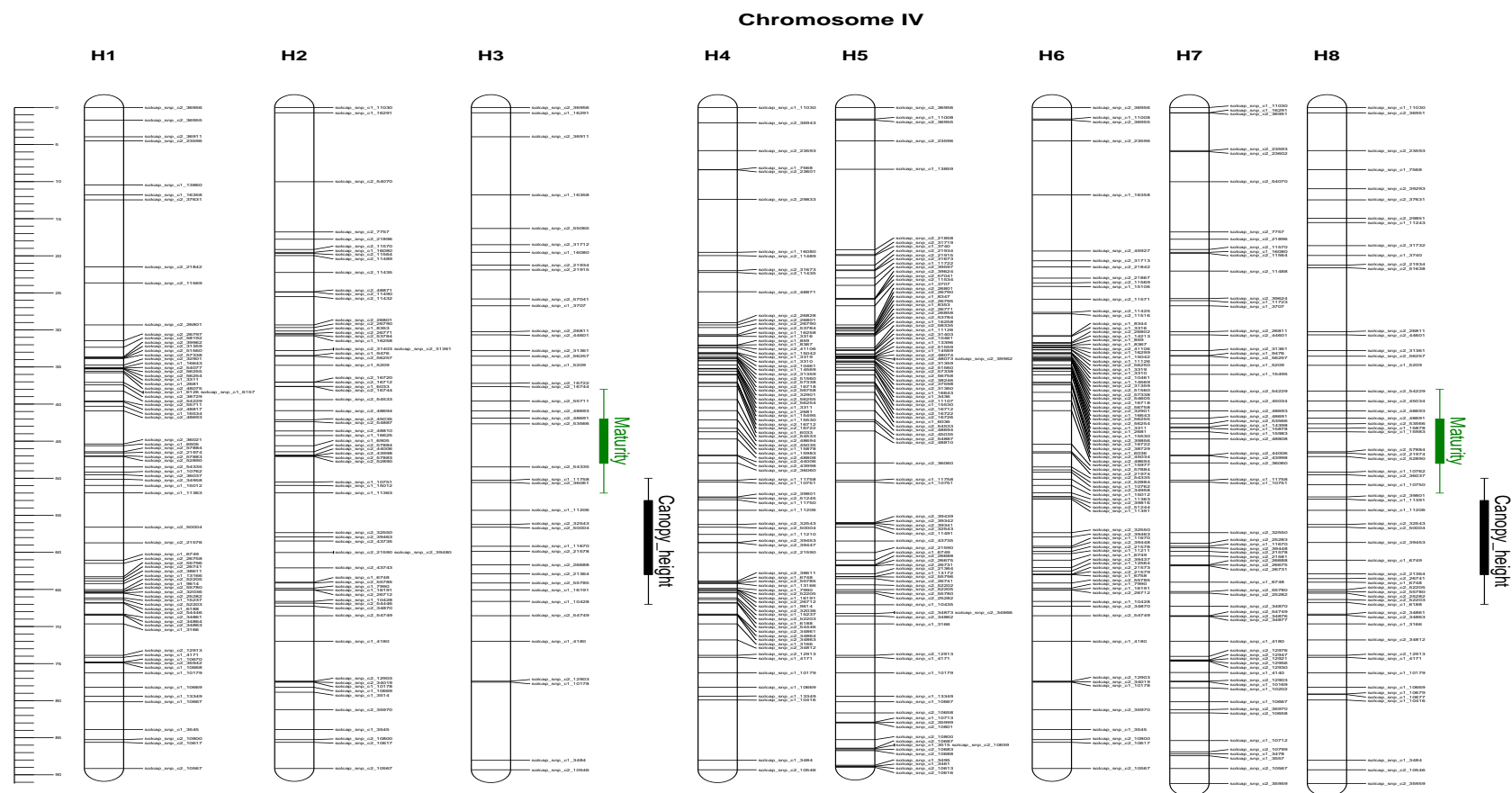


Figure 8.11 Linkage maps of Lady Rosetta (H1 to H4) and Cara (H5 to H8) chromosome IV. The green and black boxplots correspond to the QTL position for maturity and canopy height, respectively. In the boxplot, whiskers correspond to the two LOD support interval and the solid box represents 95% confidence interval.

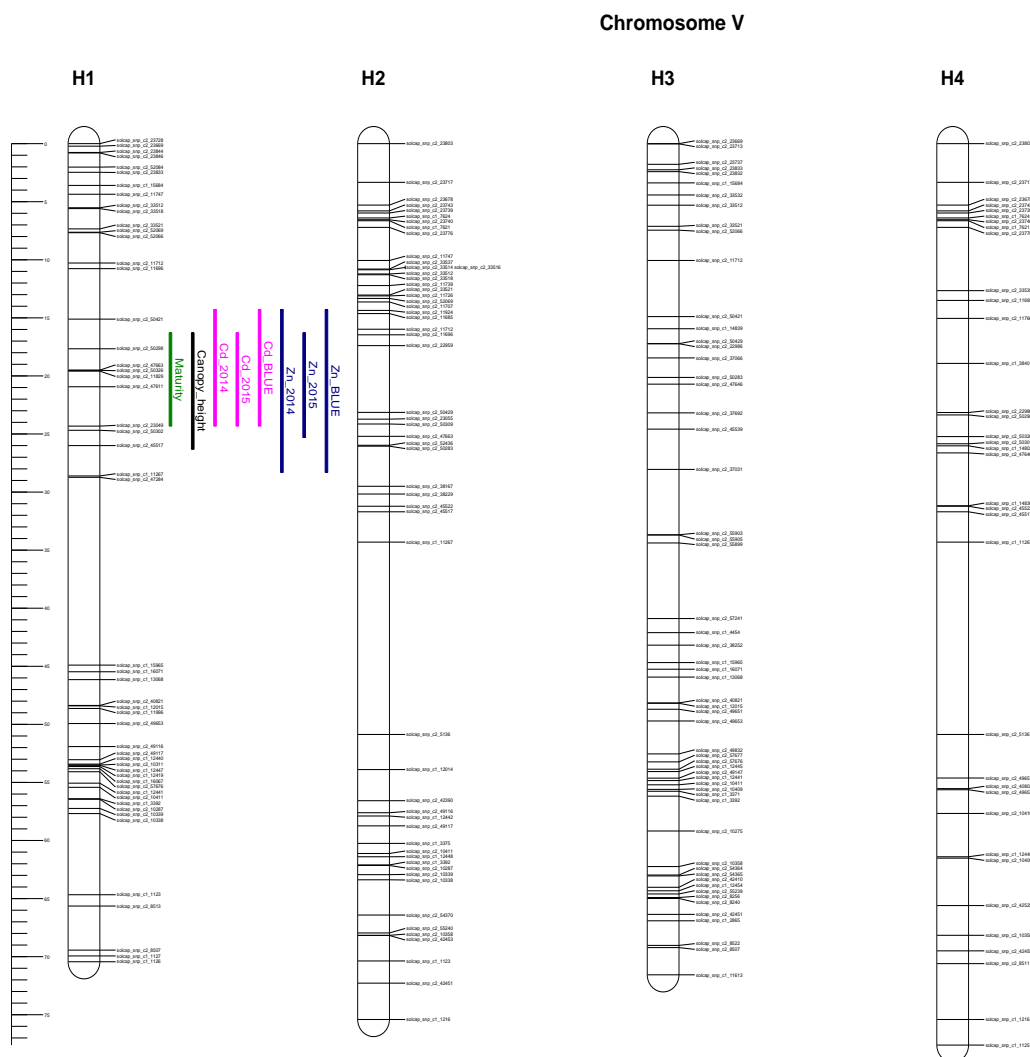


Figure 8.11 Linkage maps of Lady Rosetta chromosome V (H1 to H4). The green, black, blue and rose boxplots correspond to the QTL position for maturity, canopy height, tuber Cd and Zn concentration, respectively. In the boxplot, whiskers correspond to the two LOD support interval and the solid box represents 95% confidence interval.

Chromosome VI

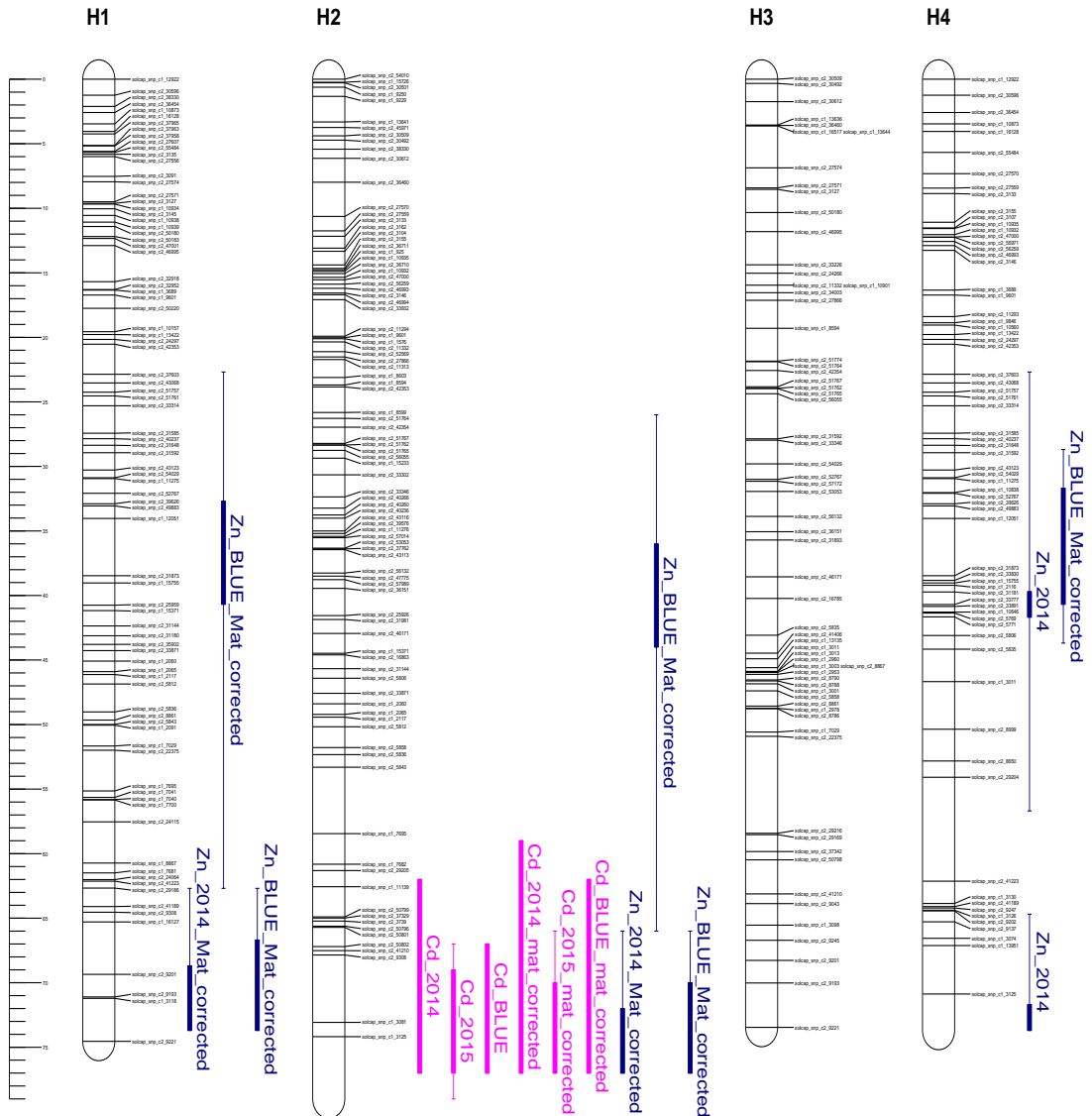


Figure 8.11 Linkage maps of Lady Rosetta chromosome VI (H1 to H4). The blue and rose boxplots correspond to the QTL position for tuber Cd and Zn concentration, respectively. In the boxplot, whiskers correspond to the two LOD support interval and the solid box represents 95% confidence interval.

Chromosome VII

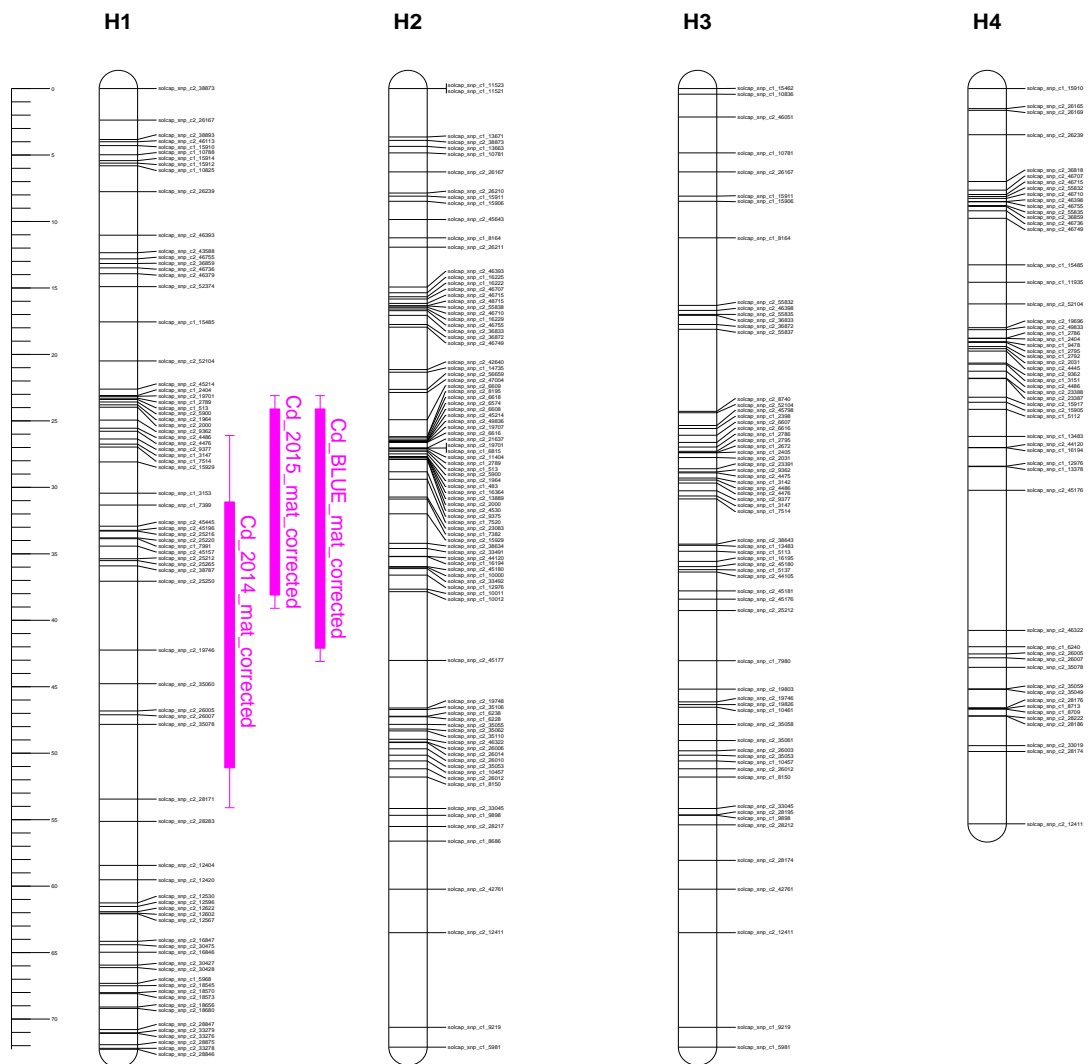


Figure 8.11 Linkage map of Lady Rosetta chromosome VII (H1 to H4). The rose boxplots represent the QTL position for tuber Cd concentration. In the boxplot, whiskers correspond to the two LOD support interval and the solid box represents the 95% confidence interval.

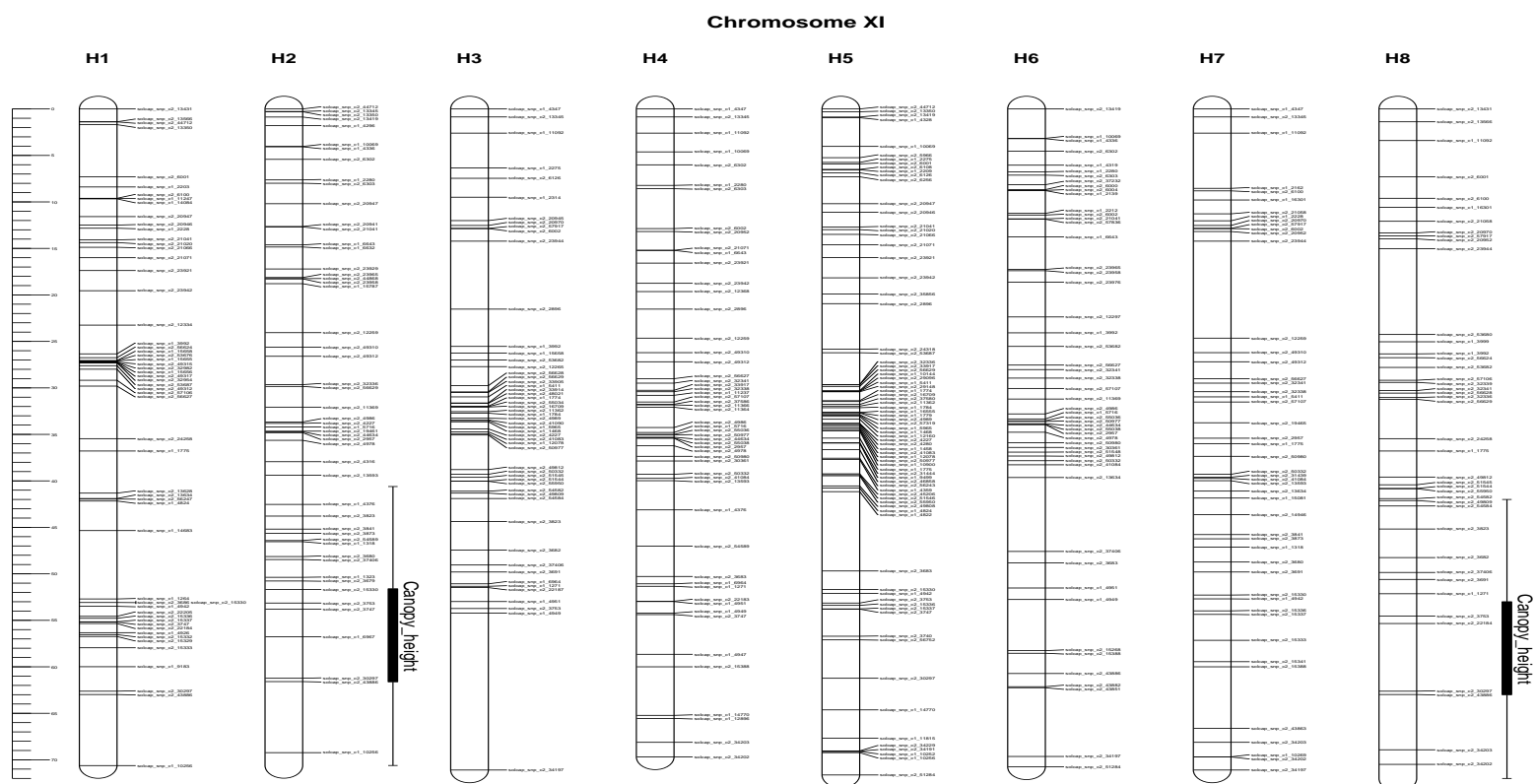


Figure 8.11 Linkage maps of Lady Rosetta (H1 to H4) and Cara (H5 to H8).chromosome XI. The black boxplots correspond to the QTL position for canopy height. In the boxplot, whiskers correspond to the two LOD support interval and the solid box represents 95% confidence interval.

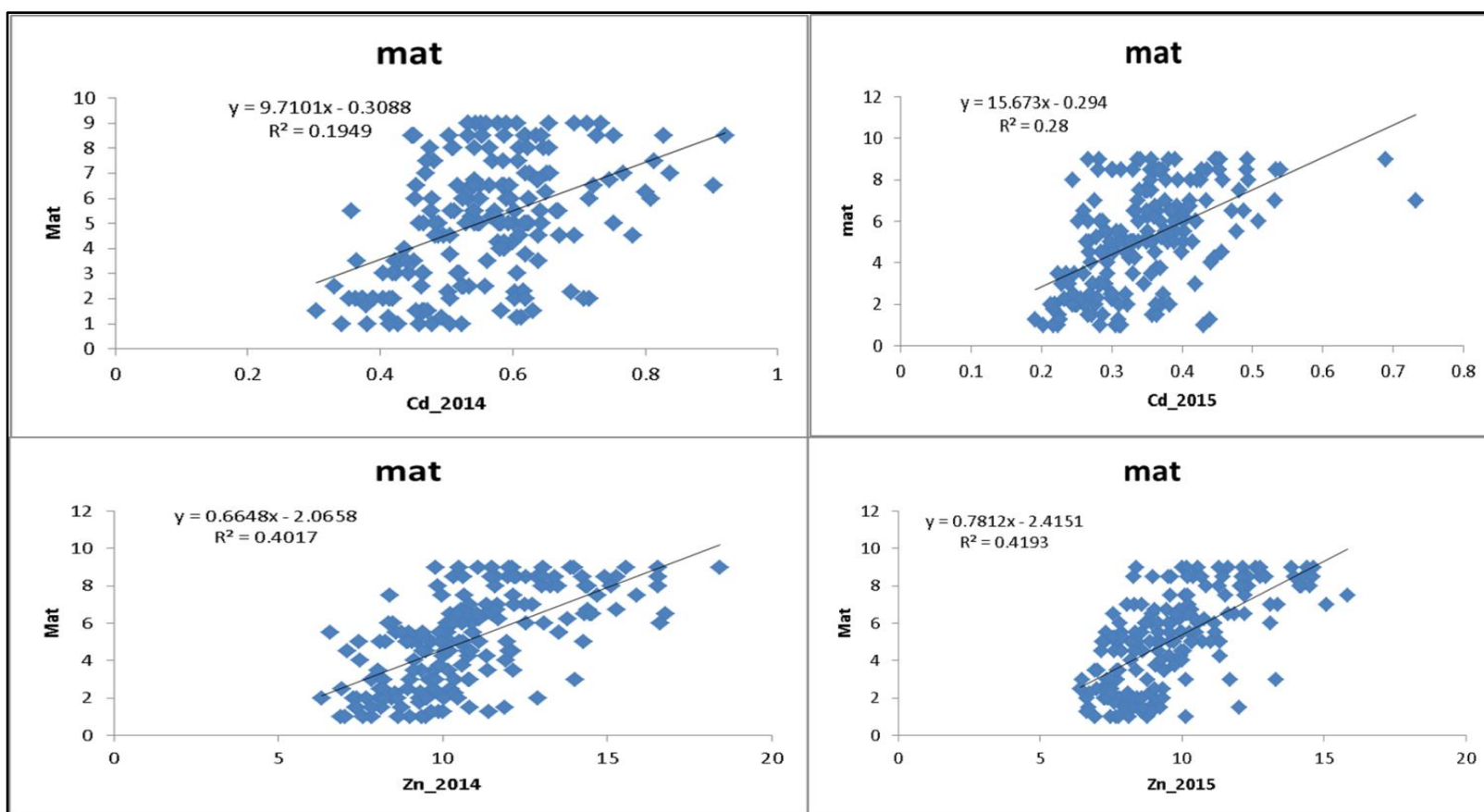


Figure 8.12 Correlation between maturity and tuber Cd and Zn concentration in 188 F1 genotypes derived from Cara and Lady Rosetta. Mat, maturity score; Cd_2014, Cd concentration data from 2014; Cd_2015, Cd concentration data from 2015; Cd_2014, Zn concentration data from 2014; Zn_2015, Zn concentration data from 2015.